PCT REQUEST ABD-001-PCT

Original (for **SUBMISSION**) - printed on 10.03.2000 03:46:26 PM

0	**For receiving Office use only**	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	**Form - PCT/RO/101 PCT Request**	
0-4-1	Prepared using	PCT-EASY Version 2.90 (updated 15.12.1999)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	European Patent Office (EPO) (RO/EP)
0-7	**Applicant's or agent's file reference**	ABD-001-PCT
I	**Title of invention**	SESQUITERPENOID SYNTHASE GENES AND THEIR USE FOR INFLUENCING BITTERNESS AND RESISTANCE IN PLANTS
II	**Applicant**	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States
II-4	Name	RESEARCH INSTITUTE FOR AGROBIOLOGY AND SOIL FERTILITY (AB-DLO)
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II-7	State of residence	NL
II-8	Telephone No.	+31 317 475881
II-9	Facsimile No.	+31 317 423110
III-1	**Applicant and/or Inventor**	
III-1-1	This person is:	applicant and inventor
III-1-2	Applicant for	US only
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III-1-6	State of nationality	NL
III-1-7	State of residence	NL

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III-2	**Applicant and/or inventor**	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	KODDE, Jan
III-2-5	Address:	Van Doesburglaan 28 NL-6708 MC Wageningen Netherlands
III-2-6	State of nationality	NL
III-2-7	State of residence	NL
III-3	**Applicant and/or inventor**	
III-3-1	This person is:	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	DE KRAKER, Jan-Willem
III-3-5	Address:	Treubstraat 31 NL-6702 BA Wageningen Netherlands
III-3-6	State of nationality	NL
III-3-7	State of residence	NL
IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	DE CLERCQ, Ann
IV-1-2	Address:	Ann De Clercq & Co B.V.B.A. Brandstraat 100 B-9830 Sint-Martens-Latem Belgium
IV-1-3	Telephone No.	+32 9 280 23 40
IV-1-4	Facsimile No.	+32 9 280 23 45
IV-1-5	e-mail	ann.declercq@pophost.eunet.be
V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW SD SL SZ TZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT

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V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AL AM AT AU AZ BA BB BG BR BY CA CH&LI CN CR CU CZ DE DK DM EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW
V-5	**Precautionary Designation Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.	
V-6	**Exclusion(s) from precautionary designations**	NONE
VI-1	**Priority claim of earlier regional application**	
VI-1-1	Filing date	12 March 1999 (12.03.1999)
VI-1-2	Number	99870046.2
VI-1-3	Regional Office	EP
VI-2	**Priority document request** The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)
VII-2	**Request to use results of earlier search; reference to that search**	
VII-2-1	Date	06 September 1999 (06.09.1999)
VII-2-2	Number	EP 99870046.2
VII-2-3	Country (or regional Office)	EP

VIII	**Check list**		
VIII-1	Request	number of sheets: 4	electronic file(s) attached: -
VIII-2	Description	44	-
VIII-3	Claims	6	-
VIII-4	Abstract	1	abstract.txt
VIII-5	Drawings	22	-
VIII-7	TOTAL	77	
	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-16	PCT-EASY diskette	-	diskette

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VIII-18	Figure of the drawings which should accompany the abstract	
VIII-19	Language of filing of the international application	English
IX-1	Signature of applicant or agent	
IX-1-1	Name (LAST, First)	DE CLERCQ, Ann

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/EP 00/02130

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/52 C12N9/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLBY S M ET AL.: "Germacrene C synthase from Lycopersicum esculentum cv. VFNT cherry tomato: cDNA isolation, characterization, and bacterial expression of the multiple product sesquiterpene cyclase" PROCEEDING OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 95, March 1998 (1998-03), pages 2216-2221, XP002112685	1,10
Y	abstract; figures 2-5 page 2217 page 2219, paragraph 4 page 2220, paragraph 3 -page 2221, paragraph 1	2-9

-/—

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 2000	21/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
ABD-001-PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/02130	10/03/2000	12/03/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant RESEARCH INSTITUTE FOR AGROBIOLOGY AND SOIL FERTIL		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05/10/2000	01.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Marinoni, J-C Telephone No. +49 89 2399 8563

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/02130

I. Basis of th report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-44 as originally filed

Claims, No.:

1-39 as originally filed

Drawings, sheets:

1/22-22/22 as originally filed

Sequence listing part of the description, pages:

1-11, filed with the demand

2. With regard to the **language,** all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/02130

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire International application.

   ☒ claims Nos. 16, 21, 22, 30-36, 39 (all partially).

because:

   ☐ the said International application, or the said claims Nos. relate to the following subject matter which do s not require an international preliminary examination (*specify*):

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☒ the claims, or said claims Nos. 16, 21, 22, 30-36, 39 (all partially) are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/02130

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 2-32, 37-39
	No:	Claims 1, 33-36
Inventive step (IS)	Yes:	Claims 25-27
	No:	Claims 1-24, 28-32, 37-39
Industrial applicability (IA)	Yes:	Claims 1-39
	No:	Claims none

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/EP00/02130

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. **Claim 1** is directed to nucleic acids defined by the fact that they encode "a protein or a polypeptide having the biological activity of a germacrene A synthase". Such a wording unduly tries to expand the scope of the claim to
   (i) undefined proteins not related to the proteins of SEQ ID No 7 or 8 of the present application and having germacrene A synthase activity,
   (ii) but also to proteins which would possess an enzymatic activity (but not germacrene A synthase activity) which would also be possibly displayed by proteins having germacrene A synthase activity.

   Such proteins are not sufficientcly disclosed (Articles 5 and 6 PCT).

2. **Claim 21** refers to a DNA encoding a RNA or protein which "induces, increases or decreases the expression of germacrene A synthase". The wording of the claim partially comprises the DNAs of SEQ ID No 3 and 4 and the complementary sequences thereof for which support can be found (or derived) from the application as filed. However the wording of the claim also includes those unknown/undefined proteins (and DNAs and RNAs encoding them) which alter *in vivo* the expression of germacrene synthase: the provision of such proteins (and the corresponding DNAs or RNAs) is not supported by the description and thus not clear (Article 6 PCT). Consequently, the characterization of said proteins imposes an undue burden on the skilled person wanting to put the invention into practive over its entire range, *i.e.* the invention is not sufficiently disclosed (Article 5 PCT).
   The same applies to **claims 22 and 30-36** also partially.

   The same objection applies *mutatis mutandis* to the subject-matter of **claim 16** partially.

3. **Claim 39** is partially directed to a process for producing a plant with reduced bitterness comprising reducing the production of a sesquiterpenoid lactone derived from germacrene A. This wording does not exclude that the function of (unknown or unforessen) enzymes of the metabolic pathway of said undefined

sesquiterpenoid lactones could be altered. However, these enzymes are not defined (no support by the description, Article 5 PCT) and therefore this part of **claim 39** is not sufficiently disclosed, contrary to Article 5 PCT.

Re Item IV

Lack of unity of invention

1. The separate inventions/groups of invention are:
   (i) the DNA of SEQ ID No 3 and the polypeptide of SEQ ID No 7, methods and processes, plants, probes and primers, etc... related thereto (**claims 1-39** all partially).
   (ii) the DNA of SEQ ID No 4 and the polypeptide of SEQ ID No 8, methods and processes, plants, probes and primers, etc... related thereto (**claims 1-39** all partially).

2. They are not so linked as to form a single general inventive concept (Rule 13.1 PCT) for the following reasons:
   (a) The subject-matter of independent **claim 1** is already known (see the grounds for this objection).
   (b) The subject-matter of **claims 1-10** is not inventive (see the grounds for this objection).
   (c) The technical relationship involving one or more of the same or corresponding special technical features in the sense of Rule 13.2 PCT (here a nucleic acid encoding a protein having germacrene A synthase activity) between the subject-matter of the indentified groups of inventions (i) and (ii) is neither new nor inventive.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:
   D1: COLBYet al. 'Germacrene C synthase from *Lycopersicum esculentum* cv. VFNT cherry tomato: cDNA isolation, characterization, and bacterial expression of the multiple product sesquiterpene cyclase', PROC. NATL.

ACAD. SCI. USA, Vol. 95, March 1998, pages 2216-2221

D2: DE KRAKER et al. '(+)-Germacrene A biosynthesis: The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory', PLANT PHYSIOLOGY, Vol. 117, No. 4, pages 1381-1392

2. **Claim 1** is directed to a nucleic acid sequence encoding a polypeptide having the biological activity of a germacrene A synthase (see **Item VIII-1**).
D1 discloses a cDNA encoding a protein having germacrene C and germacrene A synthase activity (see the abstract, lines 17-20; page 2219, right column, line 57).
Therefore, the subject-matter of **claim 1** does not meet the requirements of Article 33(2) PCT concerning novelty.

3. **D2** discloses the purification of a protein of chicory that has germacrene A synthase activity. The purification to homogeneity, amino acid sequencing and cDNA cloning of the protein appear to be matters of common laboratory procedure which does not involve a particular prejudice.
Therefore, the subject-matter of **claim 1**, and consequently of **claims 2-10**, does not meet the requiremenst of Article 33(3) PCT concerning inventive step.

4. **Claim 11** is directed to a process for producing a plant with reduced bitterness by reducing the expression of an endogenous sesquiterpenoid synthase gene.
The claim lacks essential technical features (see **item VIII-4**).
D2 specifically links bitterness in plants (for example chicory, see page 1381, left column, line 4) with germacrene A synthesis. Upon reading **D2**, it is obvious to the skilled person that the reduction of germacrene A synthase expression in plants would reduce bitterness, since the accumulation of germacrene A or its derivatives is the identified cause of bitterness in some plants. In view of reducing bitterness in plants, the skilled person would use a gene construct which, upon expression in a plant, decreases the expression of germacrene A synthase, said gene construct being based upon (part(s) of) the complementary sequence of the gene encoding the protein of **D2**.
Therefore, the subject-matter of **claims 11-15** does not meet the requirements of Article 33(3) PCT concerning inventive step.

The same objection applies to the subject-matter of **claim 39**.

5. **Claim 16** is directed to a process for producing a plant with increased pest resistance.
The claim lacks essential technical features (see **item VIII-5**).
Sesquiterpenoid-based plant defence is known from the art (see **D1**, page 2216, right column, lines 5-14; page 2221, left column, last 5 lines). Therefore, upon reading **D1**, the skilled person would introduce the gene of **D1** or the gene encoding the protein of **D2** into plants in order to (possibly) obtain plants with increased resistance to pests.
Therefore, the subject-matter of **claims 16-20** does not meet the requirements of Article 33(3) PCT concerning inventive step.

6. **Claim 21** is directed to a "recombinant polynucleic acid" (see **items VIII-6**). In view of obtaining the plants by the process of claims 11-15 or 16-20, the skilled person would, without the exercise of inventive skills put the gene encoding the proteins of **D1** or **D2** or their complement under the control of a plant promoter.
Therefore, the subject-matter of **claims 21**, but also claims **22-24 and 28-32** does not meet the requirements of Article 33(3) PCT concerning inventive step.

7. An inventive step is acknowledged for those "recombinant polynucleic acids" comprising parts of SEQ ID No 3 and parts of SEQ ID No 4.
Therefore, the subject-matter of **claims 25-27** meets the requirements of Article 33(2) PCT concerning novelty and the requirements of Article 33(3) PCT concerning inventive step. However, the objections under **Item VIII-7** should be taken into consideration.

8. **Claim 33** is directed to a cell of a plant transformed with the recombinant polynucleic acid of claim 21-32. The comment under **item VIII-6 and 8** should be taken into consideration. When referring to the nucleic acid of claims 21-24 and 28-32, the subject-matter of **claims 33-36** covers plants, cells and seeds which express "naturally" the germacrene synthase gene.
Consequently, the subject-matter of **claims 33-36** does not meet the requirements of Article 33(2) PCT concerning novelty.

9. No inventive step can be acknowledged for probes and primers derived from known or non-inventive genes.

Therefore, the subject-matter of **claims 37 and 38** does not meet the requirement of Article 33(3) PCT concerning inventive step.

Re Item VIII

Certain observations on the international application

1. **Claim 1** does not meet the requirements of Article 6 PCT concerning clarity for the following reasons:
   (i) the wording of the claim tries to define a product by the result to be achieved ("encoding a polypeptide having the biological activity of a germacrene A synthase") (see the Guidelines, Ch. III, 4.7).
   (ii) the claim lacks essential technical features (contrary to Rule 6 PCT in combination with Article 6 PCT).

2. **Claim 2** refers to nucleic acid sequences encoding polypeptides having 70% protein similarity with the polypeptides having SEQ ID No. 7 ou 8.
   The figure "70%" is not supported by the description. Such a support must be of a technical character (*i.e.* examples/results of experimentation), not mere statements.
   The same objection applies *mutatis mutandis* to **claims 3, 6, 7, 15, 18, 19, 23, 25, 27, 29**.
   Furthermore, the term "similarity" cannot be used to define the relation existing between two DNA molecules (**claims 6, 7, 15, 19, 23, 25, 27**). The term "identity" should be used instead (on the term "similarity" in conjunction with protein sequences, see **item VIII-3**).

3. **Claims 2, 3, 18 and 29** define a nucleic acid sequence as encoding a protein that has at least 70% similarity to another protein. The claims are not clear (Article 6 PCT) for the reasons that the term "similarity" does not designate a true identity but a relationship between two amino acids (for example, one distinguishes groups of basic amino acids (Arg, Lys) from hydrophobic amino acids (Met, Ile, Leu, Val, Phe, Trp, etc...) from acidic amino acids (Glu, Asp.)). Therefore this term expands the scope of the claim (*i.e.* the number of possible combinations) beyond the point where a meaningful opinion can be given.

4. **Claim 11** lacks technical features which are considered to be essential for the

definition of the identified groups of inventions (Rule 6 PCT taken in combination with Article 6 PCT), *viz.* a link to the proteins of SEQ ID No. 7 and 8.
The same objection applies *mutatis mutandis* to **claims 12 and 39.**

5. The objection under **item VIII-4** applies also to **claims 16 and 17.** Furthermore, it is noted that no support of technical character appears in the description as filed for the claimed process. Consequently, the wording of **claims 16-20** is merely considered as a recitation of a desired result to be achieved (Article 6 PCT; the Guidelines, Ch. III, 4.7).

6. It is noted that the mere specification that a nucleic acid is "recombinant" does not render it novel or inventive compared to the "natural" nucleic acids disclosed in the prior art. Similarly, a plant defined only by the fact that it contains a recombinant gene is not distinguishable from the "natural" plant.

7. The multiple possibilities arising from the wording of **claims 25-27** (the conjunction of the provision of a nucleic acid sequence having 70% similarity with the use of the terms "or" and "and") renders the subject-matter for which protection is sought unclear. It appears that among the many combinations deriving from the wording of the claims, some at least are not supported by the description (Article 6 PCT) or unsufficiently disclosed (Article 5 PCT).

8. **Claim 33** defines a cell by the process used to obtain it ("transformed with..."). Novelty can only be acknowledged if the claimed product is distinguishable from the other products of the prior art.

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

DE CLERCQ, Ann
De Clercq, Brants & Partners cv
E. Gevaertdreef 10a
B-9830 Sint-Martens-Latem
BELGIQUE

Date of mailing (day/month/year)
19 September 2000 (19.09.00)

Applicant's or agent's file reference
ABD-001-PCT

IMPORTANT NOTIFICATION

International application No.
PCT/EP00/02130

International filing date (day/month/year)
10 March 2000 (10.03.00)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
DE CLERCQ, Ann
Ann De Clercq & Co B.V.B.A.
Brandstraat 100
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Belgium

State of Nationality:
State of Residence:
Telephone No.: +32 9 280 23 40
Facsimile No.: +32 9 280 23 45
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
DE CLERCQ, Ann
De Clercq, Brants & Partners cv
E. Gevaertdreef 10a
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State of Nationality:
State of Residence:
Telephone No.: +32 9 280 23 40
Facsimile No.: +32 9 280 23 45
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[X] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
H. Zhou
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 31 October 2000 (31.10.00)	
International application No. PCT/EP00/02130	**Applicant's or agent's file reference** ABD-001-PCT
International filing date (day/month/year) 10 March 2000 (10.03.00)	**Priority date** (day/month/year) 12 March 1999 (12.03.99)
Applicant BOUWMEESTER, Harro et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

05 October 2000 (05.10.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer F. Baechler
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
ABD-001-PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 02130	10/03/2000	12/03/1999
Applicant		
RESEARCH INSTITUTE FOR AGROBIOLOGY AND SOIL FERTIL		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

International Application No
PC[redacted]P 00/02130

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/52 C12N9/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLBY S M ET AL.: "Germacrene C synthase from Lycopersicum esculentum cv. VFNT cherry tomato: cDNA isolation, characterization, and bacterial expression of the multiple product sesquiterpene cyclase" PROCEEDING OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 95, March 1998 (1998-03), pages 2216-2221, XP002112685	1,10
Y	abstract; figures 2-5 page 2217 page 2219, paragraph 4 page 2220, paragraph 3 -page 2221, paragraph 1	2-9

-/--

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 2000	21/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y .	DE KRAKER, JAN-WILLEM ET AL: "(+)-Germacrene A biosynthesis: The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory." PLANT PHYSIOLOGY (ROCKVILLE), (AUG., 1998) VOL. 117, NO. 4, PP. 1381-1392., XP002112686 the whole document	2-9

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

PCT

INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

Aktenzeichen des Anmelders oder Anwalts	**WEITERES VORGEHEN**	siehe Mitteilung über die Übermittlung des internationalen Recherchenberichts (Formblatt PCT/ISA/220) sowie, soweit zutreffend, nachstehender Punkt 5
IP 3554 PCT		
Internationales Aktenzeichen	Internationales Anmeldedatum *(Tag/Monat/Jahr)*	(Frühestes) Prioritätsdatum *(Tag/Monat/Jahr)*
PCT/EP 00/ 01684	29/02/2000	06/03/1999
Anmelder		
AUDI AKTIENGESELLSCHAFT		

Dieser internationale Recherchenbericht wurde von der Internationalen Recherchenbehörde erstellt und wird dem Anmelder gemäß Artikel 18 übermittelt. Eine Kopie wird dem Internationalen Büro übermittelt.

Dieser internationale Recherchenbericht umfaßt insgesamt 2 Blätter.

[X] Darüber hinaus liegt ihm jeweils eine Kopie der in diesem Bericht genannten Unterlagen zum Stand der Technik bei.

1. **Grundlage des Berichts**

   a. Hinsichtlich der **Sprache** ist die internationale Recherche auf der Grundlage der internationalen Anmeldung in der Sprache durchgeführt worden, in der sie eingereicht wurde, sofern unter diesem Punkt nichts anderes angegeben ist.

   [ ] Die internationale Recherche ist auf der Grundlage einer bei der Behörde eingereichten Übersetzung der internationalen Anmeldung (Regel 23.1 b)) durchgeführt worden.

   b. Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** ist die internationale Recherche auf der Grundlage des Sequenzprotokolls durchgeführt worden, das

   [ ] in der internationalen Anmeldung in Schriftlicher Form enthalten ist.

   [ ] zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.

   [ ] bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.

   [ ] bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.

   [ ] Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.

   [ ] Die Erklärung, daß die in computerlesbarer Form erfaßten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

2. [ ] **Bestimmte Ansprüche haben sich als nicht recherchierbar erwiesen** (siehe Feld I).

3. [ ] **Mangelnde Einheitlichkeit der Erfindung** (siehe Feld II).

4. Hinsichtlich der **Bezeichnung der Erfindung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut von der Behörde wie folgt festgesetzt:

5. Hinsichtlich der **Zusammenfassung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut nach Regel 38.2b) in der in Feld III angegebenen Fassung von der Behörde festgesetzt. Der Anmelder kann der Behörde innerhalb eines Monats nach dem Datum der Absendung dieses internationalen Recherchenberichts eine Stellungnahme vorlegen.

6. Folgende Abbildung der **Zeichnungen** ist mit der Zusammenfassung zu veröffentlichen: Abb. Nr. 2a, 2b

   [X] wie vom Anmelder vorgeschlagen

   [ ] keine der Abb.

   [ ] weil der Anmelder selbst keine Abbildung vorgeschlagen hat.

   [ ] weil diese Abbildung die Erfindung besser kennzeichnet.

Internationales Aktenzeichen
PC[redacted] 00/01684

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 7 B21D26/02

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 7 B21D B21C

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	US 5 557 961 A (GENERAL MOTORS CORPORATION) 24. September 1996 (1996-09-24) Spalte 3, Zeile 63-67; Abbildungen 2,3,5,6	1,3
Y	Spalte 4, Zeile 61 -Spalte 5, Zeile 2 Spalte 5, Zeile 33-38	2
Y	DE 14 52 646 A (FORD MOTOR CO) 27. März 1969 (1969-03-27) Abbildung 9	2

☐ Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

☒ Siehe Anhang Patentfamilie

° Besondere Kategorien von angegebenen Veröffentlichungen :
"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist
"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist
"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)
"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht
"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist
"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden
"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist
"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
12. Juli 2000	21/07/2000

Name und Postanschrift der Internationalen Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ash, R

1

Inform[redacted]on patent family members

International Application No
PC[redacted] 00/01684

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5557961 A	24-09-1996	NONE	
DE 1452646 A	27-03-1969	NONE	

TENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
ABD-001-PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/02130	10/03/2000	12/03/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant RESEARCH INSTITUTE FOR AGROBIOLOGY AND SOIL FERTIL		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05/10/2000	01.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Marinoni, J-C Telephone No. +49 89 2399 8563

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/02130

I. Basis f the rep rt

1. With regard to the **l ments** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-44 as originally filed

Claims, No.:

1-39 as originally filed

Drawings, sheets:

1/22-22/22 as originally filed

Sequence listing part of the description, pages:

1-11, filed with the demand

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☒ furnished subsequently to this Authority in written form.

   ☒ furnished subsequently to this Authority in computer readable form.

   ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 16, 21, 22, 30-36, 39 (all partially).

because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☒ the claims, or said claims Nos. 16, 21, 22, 30-36, 39 (all partially) are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of inventi n

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2-32, 37-39
	No:	Claims	1, 33-36
Inventive step (IS)	Yes:	Claims	25-27
	No:	Claims	1-24, 28-32, 37-39
Industrial applicability (IA)	Yes:	Claims	1-39
	No:	Claims	none

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate she t

R lt m III

Non- stablishm nt of opinion with regard to n v lty, inv ntiv step and industrial applicability

1. **Claim 1** is directed to nucleic acids defined by the fact that they encode "a protein or a polypeptide having the biological activity of a germacrene A synthase". Such a wording unduly tries to expand the scope of the claim to
   (i) undefined proteins not related to the proteins of SEQ ID No 7 or 8 of the present application and having germacrene A synthase activity,
   (ii) but also to proteins which would possess an enzymatic activity (but not germacrene A synthase activity) which would also be possibly displayed by proteins having germacrene A synthase activity.

   Such proteins are not sufficientcly disclosed (Articles 5 and 6 PCT).

2. **Claim 21** refers to a DNA encoding a RNA or protein which "induces, increases or decreases the expression of germacrene A synthase". The wording of the claim partially comprises the DNAs of SEQ ID No 3 and 4 and the complementary sequences thereof for which support can be found (or derived) from the application as filed. However the wording of the claim also includes those unknown/undefined proteins (and DNAs and RNAs encoding them) which alter *in vivo* the expression of germacrene synthase: the provision of such proteins (and the corresponding DNAs or RNAs) is not supported by the description and thus not clear (Article 6 PCT). Consequently, the characterization of said proteins imposes an undue burden on the skilled person wanting to put the invention into practive over its entire range, *i.e.* the invention is not sufficiently disclosed (Article 5 PCT).

   The same applies to **claims 22 and 30-36** also partially.

   The same objection applies *mutatis mutandis* to the subject-matter of **claim 16** partially.

3. **Claim 39** is partially directed to a process for producing a plant with reduced bitterness comprising reducing the production of a sesquiterpenoid lactone derived from germacrene A. This wording does not exclude that the function of (unknown or unforessen) enzymes of the metabolic pathway of said undefined

sesquiterpenoid lactones could be altered. However, these enzymes are not defined (no support by the description, Article 5 PCT) and therefore this part of **claim 39** is not sufficiently disclosed, contrary to Article 5 PCT.

Re Item IV

Lack of unity of invention

1. The separate inventions/groups of invention are:
   (i) the DNA of SEQ ID No 3 and the polypeptide of SEQ ID No 7, methods and processes, plants, probes and primers, etc... related thereto (**claims 1-39** all partially).
   (ii) the DNA of SEQ ID No 4 and the polypeptide of SEQ ID No 8, methods and processes, plants, probes and primers, etc... related thereto (**claims 1-39** all partially).

2. They are not so linked as to form a single general inventive concept (Rule 13.1 PCT) for the following reasons:
   (a) The subject-matter of independent **claim 1** is already known (see the grounds for this objection).
   (b) The subject-matter of **claims 1-10** is not inventive (see the grounds for this objection).
   (c) The technical relationship involving one or more of the same or corresponding special technical features in the sense of Rule 13.2 PCT (here a nucleic acid encoding a protein having germacrene A synthase activity) between the subject-matter of the indentified groups of inventions (i) and (ii) is neither new nor inventive.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:
   D1: COLBYet al. 'Germacrene C synthase from *Lycopersicum esculentum* cv. VFNT cherry tomato: cDNA isolation, characterization, and bacterial expression of the multiple product sesquiterpene cyclase', PROC. NATL.

ACAD. SCI. USA, Vol. 95, March 1998, pages 2216-2221

D2: DE KRAKER et al. '(+)-Germacrene A biosynthesis: The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory', PLANT PHYSIOLOGY, Vol. 117, No. 4, pages 1381-1392

2. **Claim 1** is directed to a nucleic acid sequence encoding a polypeptide having the biological activity of a germacrene A synthase (see **Item VIII-1**).
D1 discloses a cDNA encoding a protein having germacrene C and germacrene A synthase activity (see the abstract, lines 17-20; page 2219, right column, line 57).
Therefore, the subject-matter of **claim 1** does not meet the requirements of Article 33(2) PCT concerning novelty.

3. **D2** discloses the purification of a protein of chicory that has germacrene A synthase activity. The purification to homogeneity, amino acid sequencing and cDNA cloning of the protein appear to be matters of common laboratory procedure which does not involve a particular prejudice.
Therefore, the subject-matter of **claim 1**, and consequently of **claims 2-10**, does not meet the requiremenst of Article 33(3) PCT concerning inventive step.

4. **Claim 11** is directed to a process for producing a plant with reduced bitterness by reducing the expression of an endogenous sesquiterpenoid synthase gene.
The claim lacks essential technical features (see **item VIII-4**).
D2 specifically links bitterness in plants (for example chicory, see page 1381, left column, line 4) with germacrene A synthesis. Upon reading **D2**, it is obvious to the skilled person that the reduction of germacrene A synthase expression in plants would reduce bitterness, since the accumulation of germacrene A or its derivatives is the identified cause of bitterness in some plants. In view of reducing bitterness in plants, the skilled person would use a gene construct which, upon expression in a plant, decreases the expression of germacrene A synthase, said gene construct being based upon (part(s) of) the complementary sequence of the gene encoding the protein of **D2**.
Therefore, the subject-matter of **claims 11-15** does not meet the requirements of Article 33(3) PCT concerning inventive step.

The same objection applies to the subject-matter of **claim 39**.

5. **Claim 16** is directed to a process for producing a plant with increased pest resistance.
The claim lacks essential technical features (see **item VIII-5**).
Sesquiterpenoid-based plant defence is known from the art (see **D1**, page 2216, right column, lines 5-14; page 2221, left column, last 5 lines). Therefore, upon reading **D1**, the skilled person would introduce the gene of **D1** or the gene encoding the protein of **D2** into plants in order to (possibly) obtain plants with increased resistance to pests.
Therefore, the subject-matter of **claims 16-20** does not meet the requirements of Article 33(3) PCT concerning inventive step.

6. **Claim 21** is directed to a "recombinant polynucleic acid" (see **items VIII-6**). In view of obtaining the plants by the process of claims 11-15 or 16-20, the skilled person would, without the exercise of inventive skills put the gene encoding the proteins of **D1** or **D2** or their complement under the control of a plant promoter.
Therefore, the subject-matter of **claims 21**, but also claims **22-24 and 28-32** does not meet the requirements of Article 33(3) PCT concerning inventive step.

7. An inventive step is acknowledged for those "recombinant polynucleic acids" comprising parts of SEQ ID No 3 and parts of SEQ ID No 4.
Therefore, the subject-matter of **claims 25-27** meets the requirements of Article 33(2) PCT concerning novelty and the requirements of Article 33(3) PCT concerning inventive step. However, the objections under **Item VIII-7** should be taken into consideration.

8. **Claim 33** is directed to a cell of a plant transformed with the recombinant polynucleic acid of claim 21-32. The comment under **item VIII-6 and 8** should be taken into consideration. When referring to the nucleic acid of claims 21-24 and 28-32, the subject-matter of **claims 33-36** covers plants, cells and seeds which express "naturally" the germacrene synthase gene.
Consequently, the subject-matter of **claims 33-36** does not meet the requirements of Article 33(2) PCT concerning novelty.

9. No inventive step can be acknowledged for probes and primers derived from known or non-inventive genes.

Therefore, the subject-matter of **claims 37 and 38** does not meet the requirement of Article 33(3) PCT concerning inventive step.

Re Item VIII

Certain observations on the international application

1. **Claim 1** does not meet the requirements of Article 6 PCT concerning clarity for the following reasons:
   (i) the wording of the claim tries to define a product by the result to be achieved ("encoding a polypeptide having the biological activity of a germacrene A synthase") (see the Guidelines, Ch. III, 4.7).
   (ii) the claim lacks essential technical features (contrary to Rule 6 PCT in combination with Article 6 PCT).

2. **Claim 2** refers to nucleic acid sequences encoding polypeptides having 70% protein similarity with the polypeptides having SEQ ID No. 7 ou 8.
   The figure "70%" is not supported by the description. Such a support must be of a technical character (*i.e.* examples/results of experimentation), not mere statements.
   The same objection applies *mutatis mutandis* to **claims 3, 6, 7, 15, 18, 19, 23, 25, 27, 29**.
   Furthermore, the term "similarity" cannot be used to define the relation existing between two DNA molecules (**claims 6, 7, 15, 19, 23, 25, 27**). The term "identity" should be used instead (on the term "similarity" in conjunction with protein sequences, see **item VIII-3**).

3. **Claims 2, 3, 18 and 29** define a nucleic acid sequence as encoding a protein that has at least 70% similarity to another protein. The claims are not clear (Article 6 PCT) for the reasons that the term "similarity" does not designate a true identity but a relationship between two amino acids (for example, one distinguishes groups of basic amino acids (Arg, Lys) from hydrophobic amino acids (Met, Ile, Leu, Val, Phe, Trp, etc...) from acidic amino acids (Glu, Asp.)). Therefore this term expands the scope of the claim (*i.e.* the number of possible combinations) beyond the point where a meaningful opinion can be given.

4. **Claim 11** lacks technical features which are considered to be essential for the

definition of the identified groups of inventions (Rule 6 PCT taken in combination with Article 6 PCT), *viz.* a link to the proteins of SEQ ID No. 7 and 8.
The same objection applies *mutatis mutandis* to **claims 12 and 39**.

5. The objection under **item VIII-4** applies also to **claims 16 and 17**. Furthermore, it is noted that no support of technical character appears in the description as filed for the claimed process. Consequently, the wording of **claims 16-20** is merely considered as a recitation of a desired result to be achieved (Article 6 PCT; the Guidelines, Ch. III, 4.7).

6. It is noted that the mere specification that a nucleic acid is "recombinant" does not render it novel or inventive compared to the "natural" nucleic acids disclosed in the prior art. Similarly, a plant defined only by the fact that it contains a recombinant gene is not distinguishable from the "natural" plant.

7. The multiple possibilities arising from the wording of **claims 25-27** (the conjunction of the provision of a nucleic acid sequence having 70% similarity with the use of the terms "or" and "and") renders the subject-matter for which protection is sought unclear. It appears that among the many combinations deriving from the wording of the claims, some at least are not supported by the description (Article 6 PCT) or unsufficiently disclosed (Article 5 PCT).

8. **Claim 33** defines a cell by the process used to obtain it ("transformed with..."). Novelty can only be acknowledged if the claimed product is distinguishable from the other products of the prior art.

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(54) Title: SESQUITERPENOID SYNTHASE GENES AND THEIR USE FOR INFLUENCING BITTERNESS AND RESISTANCE IN PLANTS

(57) Abstract

This invention relates to the use of sesquiterpenoid synthase genes, particularly genes encoding germacrene A synthase, to modulate (i.e. repress, induce or increase) the expression or activity of sesquiterpenoid synthases in plants, so as to directly or indirectly influence taste, the production of sesquiterpene lactones, and/or resistance against insects, nematodes, micro–organisms and vertebrate herbivores in the plant.

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Title of the invention

Sesquiterpenoid synthase genes and their use for influencing bitterness and resistance in plants

Field of the invention

This invention relates to the use of sesquiterpenoid synthase genes, particularly genes encoding germacrene A synthase, to modulate (i.e. repress, induce or increase) the expression or activity of sesquiterpenoid synthases in plants, so as to influence sesquiterpenoid production of these plants. More particularly, the invention is directed to the inhibition of sesquiterpenoid synthase expression, reducing the production of sesquiterpene lactones in the plant. Particularly, in crops where these sesquiterpene lactones are associated with a bitter taste, such as chicory (*Cichorium intybus L.*), the invention can be used to obtain plants or plant parts that are less bitter. More particularly, this is achieved by reducing gene expression of germacrene A synthase by anti-sensing or co-suppression. Other aspects of this invention relate to the use of sesquiterpenoid synthase genes, more particularly genes encoding germacrene A synthase, to obtain increased resistance against insects, nematodes or micro-organisms in plants, to obtain increased formation of sesquiterpene lactones with attractive, e.g. medicinal, properties, and to obtain increased formation of germacrene A derived flavor and fragrance compounds or phytoalexins. This invention also relates to plant cells and plants transformed with one or more transgenes, which results in the modulation of activity of a sesquiterpenoid synthase therein.

All documents cited are incorporated herein by reference.

Background of the Invention

The sprouts of chicory (*Cichorium intybus L.*), known as the Belgian endive, are characterized by their slightly bitter taste, which is a limiting factor to its commercial value as food crop. The taproots of chicory, which are even more bitter, were used in former days as a coffee substitute. Because of their bitterness these roots are not very well suited for use as cattle feed and are mainly regarded as a waste product of chicory cultivation. Chicory roots of specific varieties have been demonstrated to be an interesting source for inulin and/or high-fructose syrup for which special extraction procedures have been developed (Perschak and Wolfslehner, *Zuckerind.* **115**(6):466-470, 1990); nevertheless, production of fructose from chicory roots requires the removal of the bitter taste in the course of the extraction procedure.

The bitter constituents of chicory and other vegetables have been associated with sesquiterpene lactones, more particularly the guianolides lactucin, 8-deoxylactucin and lactupicrin (van Beek et al. *J. Agric. Food Chem.* **38**: 1035-1038, 1990; Price et al. *J. Sci. Food Agric.* **53**: 185-192, 1990). Other sesquiterpene lactones identified in chicory are eudesmanolides, and germacranolides (Seto et al., *Chem. Pharm. Bull.* **36**:2423-2429,1988). The sesquiterpenoids belong to a very large family of plant products, the terpenoids, which have been associated with a variety of biological functions mainly related to plant-plant, plant-insect and plant-pathogen interactions. The production of terpenoids is based on a common biosynthetic pathway after which specific enzymes or synthases lead to the individual terpenoid structures.

The initial step of this pathway involves the fusion of three molecules of acetyl CoA to produce the C6 compound 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is reduced to mevalonate by HMG-reductase, and mevalonate is in turn phosphorylated by two kinases, mevalonate- and phosphomevalonate kinase, to form 5-pyrophosphomevalonate. Pyrophosphomevalonate decarboxylase then converts the latter into isopentenyl pyrophosphate (IPP), which represents the first "active" isoprene unit or building block. Alternatively, IPP can be produced via the newly discovered mevalonate-independent deoxyxylulose pathway (Rohmer, In

Comp. Nat. Prod. Chem. Vol. 2, ed. by D. Cane, Pergamon, 1999). After isomerization of IPP to dimethylallyl pyrophosphate (DMAPP), prenyl pyrophosphate co-substrates are added to this basic unit to form longer chains. Two diphosphorylated building blocks generate geranyl diphosphate (GDP), a linear C10 intermediate, that can be converted into linear or cyclic products representing the monoterpenes. Addition of a third unit of IPP generates farnesyl diphosphate (FDP) from which the sesquiterpenoids are derived. The addition of one more IPP unit generates geranylgeranyl diphosphate (GGDP), the precursor for diterpenes, carotenoids, etc. The conversion of GDP, FDP and GGDP into terpenoid products is realized by monoterpene, sesquiterpene and diterpene synthases respectively, which give rise to stable end products or substrates for other enzymes catalyzing secondary transformations. It is assumed that the sesquiterpene lactones originate from a germacrane precursor that is formed from FDP by a germacrane synthase. The germacrane precursor is further cyclized to either a guiane skeleton (of the guianolides) or a eudesmane skeleton (of the eudesmanolides). This germacrane precursor has recently been identified as (+)-germacrene A (De Kraker et al *Plant Physiol.* **117:** 1381-1392, 1998).

Other vegetables, such as lettuce (*Lactuca salva and L. sativa*), radicchio (*Cichorium intybus*), endive (*Cichorium endivia*), and artichoke (*Cynara scolymus*) have also been demonstrated to contain sesquiterpene lactones as bitter constituents (Price et al., 1990, above; Herrmann K., *Z. Lebensm. Unters. Forsch.* **167**:262-273, 1967).

Examples of sesquiterpenoids associated with bitter taste are cnicin (from *Cnicus benedictus*), absinth (from the wormwood, *Artemisia absintha L.*), alantolactone and isoalantolactone (from *Inula helenium* roots) and helenalin (from sneezeweed, *Helenium autumnale*) (Fischer N., *Methods in Plant Biochemistry* **7**:187-211, 1991).

Several sesquiterpenoids have been described to have an anti-feedant activity on herbivorous insects and vertebrate herbivores. Examples of these are tenulin (from *Helenium amarum*; Arnason et al., *Journal of Natural products*, **50(4)**: 690-695, 1987) helenalin (from sneezeweed, *Helenium autumnale*), parthenin (from *Parthenium histerophorus*) (Picman A. and

Picman J., *Biochemical systematics and Ecology*, **12(1)**: 89-93, 1984) and linifolin A (Nawrot et al., *Prace Naukowe/OR*, **24**: 27, 1982). Many sesquiterpene lactones have been shown to possess pharmacological [parthenolide from feverfew (*Tanacetum parthenium*) has an anti-migraine effect (Hewlett et al., *Journal of the Chemical Society Perkin Transactions 1*, **16**: 1979-1986, 1996)], as well as anti-fungal, anti-bacterial, anti-protozoan, schistomicidal and molluscidal activities (Picman, *Biochemical Systematics and Ecology* **14(3):** 255-281, 1978). (-)-Germacrene A has been identified as the alarm pheromone in aphids (Bowers et al., *Science* **196**:680-681, 1976). Also, germacrene A has been postulated to be an intermediate in the formation of the important flavor compound nootkatone (Croteau and Karp, In *Perfumes: art, science and technology*, ed. by P.M. Müller and D. Lamparsky, Elsevier Science Publishers LTD, England, 1991), as well as an (enzyme-bound) intermediate in the biosynthesis of phytoalexins such as aristolochene, 5-epi-aristolochene, capsidiol, debneyol, and vetispiradiene (Back and Chappell, *J. Biol. Chem.* **270(13):** 7375-7381, 1995; Whitehead et al., *Phytochemistry* **28(3):** 775-779, 1989).

The biological structure and known functions of a large number of sesquiterpenoid lactones as well as the different methods by which they can be isolated is described in the review by Fischer (*Methods in Plant Biochemistry* **7**:187-211, 1991).

McGarvey and Croteau (*The Plant Cell* **7**:1015-1026, 1995) give an overview of the biosynthetic pathways of terpenoids and their regulation.

Chappel (*Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **46**:521-47, 1995) reviews the biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Molecular comparison of a monoterpene, a sesquiterpene and a diterpene synthase demonstrates a strong similarity in gene-organization and in amino acid sequence within domains.

A number of genes involved in sesquiterpenoid biosynthesis have been isolated, of which some examples are given:

Two independent cDNA clones encoding 5-epi-aristolochene synthase (EAS) from tobacco have been isolated and characterized by Facchini and Chappell (*Proc Natl Acad. Sci. USA*, **89**:11088-11092, 1992). The cDNA encoding farnesyl diphosphate synthase was cloned and analyzed for *Arabidopsis*

thaliana by Delourme et al. (*Plant Molec. Biol.*, **26**:1867-1873, 1994) and for *Artemisia annua* by Matsushita et al. (*Gene*, **172**:207-209, 1996). Back and Chappell described the cloning and bacterial expression of vetispiradiene synthase found in *Hyoscyamus muticus* (1995, above). Molecular comparison of this sequence with that of tobacco EAS displayed identical intron-exon organization of the gene and strong sequence similarities, which is suggested to be reflective of the conservation of several partial reactions common to both enzymes (Back and Chapell, *Proc. Natl. Acad. Sci. USA*, **93**:6841-6845, 1996).

WO 9715584 describes the use of S-linalool synthase, an acyclic monoterpene synthase, in the genetic engineering of scent production.

The use of the limonene (monoterpene) cyclase in the control of corn rootworm, by inserting a nucleotide sequence coding for limonene cyclase into the plants is described in WO 9637102.

The inactivation of endogenous genes using either sense or anti-sense transgene constructs, has been demonstrated to be successful (Mol, J.N.M. et al., In *Homologous recombination and gene silencing in plants*, ed. by J. Paszkowski, Kluwer Academic Publishers, the Netherlands, 1994; Hamilton, A.J. et al., *Current Topics in Microbiology and Immunology* **197**, 77-89, 1995; Bourque J., *Plant Science*, 105:125-149, 1995; Cannon M. et al., *Plant Molecular Biology* **15**:39-47, 1990; Smith C.J.S. et al., *Molecular and General Genetics*, **224**:477-481, 1990). Also the inactivation of two non-homologous endogenous genes using a single sense gene construct has been reported (Seymour et al., *Plant Molecular Biology* **23**:1-9, 1993). The inactivation of an endogenous gene using constructs encoding ribozymes targeting endogenous genes is described by Haselhoff and Gerlach (*Nature* **334**:585-591, 1988) and in WO 89/05852.

Alternative methods have been described for decreasing endogenous gene expression. For instance, direct modulation of the endogenous gene using the chimeric RNA-DNA oligonucleotide technology. The method is based on the construction of a chimeric RNA-DNA oligonucleotide in duplex conformation with double hairpin caps on the ends, of which the sequence is

designed so as to align with the sequence wherein a mutation is targeted and so as to contain the desired nucleotide change and subsequent introduction of the chimeric oligonucleotide in the cell. A detailed description of this method and its efficiency for bringing mutations into endogenous genes is described by Cole-Strauss et al. (*Science*, **273**:1386-1389, 1996) and in patent n° US patent number 5,565,350.

Suppression of endogenous gene activity can also be achieved by introducing transgenes encoding inhibitors of the enzymatic gene product. Modulation of physiological functions using recombinant immunoglobulins is reviewed by Conrad and Fiedler (*Plant Mol. Biol.* **38**:101-109, 1998).

The aim of the present invention is to provide a polynucleic acid sequence encoding a sesquiterpenoid synthase, more particularly a germacrene A synthase.

Another aim of the present invention is to provide a process for producing a plant with modified sesquiterpenoid synthase activity.

Another aim of the present invention is to provide a process for producing a plant or plant parts with an increased content of germacrene A or sesquiterpene lactone metabolites thereof.

Another aim of the present invention is to provide a process for producing a plant or plant parts with reduced bitterness.

Another aim of the present invention is to provide a recombinant polynucleic acid encoding germacrene A synthase.

Another aim of the present invention is to provide a plant cell or plant, which is transformed with a recombinant polynucleic acid encoding a molecule having the biological activity of a germacrene A synthase.

Any other method for suppressing, decreasing or inducing endogenous gene expression known to the skilled man is also comprised within the content of this application.

Summary of the invention

The present invention relates to an isolated polynucleic acid encoding a protein or polypeptide having the biological activity of a germacrene A synthase. The invention further relates to an isolated DNA sequence encoding a protein or polypeptide with germacrene A synthase activity, having at least 70%, preferably at least 75% or 80%, more preferably at least 85% or 90%, most preferably at least 95%, especially preferably 100% sequence similarity with part or all of the amino acid sequence of SEQ ID NO 7 and/or SEQ ID NO 8, most preferably with the region of AA 271 to 455 of SEQ ID NO 7 or the region of AA 293 to 477 of SEQ ID NO 8. The invention further relates to an isolated polynucleic acid encoding a protein or polypeptide with germacrene A synthase activity, whereby the DNA sequence has at least 70%, preferably at least 75% or 80%, more preferably at least 85% or 90%, most preferably at least 95%, especially preferably 100% sequence similarity with all or part of the DNA sequence of SEQ ID NO 3 or SEQ ID NO 4.

The term "polynucleic acid" refers to DNA or RNA, or amplified versions thereof, or the complement thereof.

The invention further relates to a polynucleic acid encoding a protein or polypeptide having the biological activity of a germacrene A synthase comprising

(a) a sequence represented in SEQ ID NO 3 or 4, or,

(b) a sequence hybridizing with a sequence as defined in (a) or,

(c) a sequence which is redundant as a result of the degeneracy of the genetic code to a sequence under (a) or (b), or

(d) a complement of any of the sequences under (a), (b) or (c).

The term "hybridizing" refers to hybridization conditions as described in Sambrook (Molecular cloning, a laboratory manual, Cold Spring Harbor Press, 1989, page 7.52), preferably specific or stringent hybridization conditions are used.

The invention further relates to a method of producing germacrene A or sesquiterpene lactones, which method comprises expressing a polynucleic

acid having at least 70%, preferably at least 75% or 80%, more preferably at least 85% or 90%, most preferably at least 95% similarity with, especially 100% sequence similarity to the nucleotide sequence of SEQ ID NO 3 and/or SEQ ID NO 4, encoding a polypeptide having the biological activity of a germacrene A synthase, in a suitable host cell, in the presence of farnesyl diphosphate, and, optionally, isolating the germacrene A or sesquiterpene lactones thus formed.

The invention further pertains to a recombinant polynucleic acid comprising one or more DNA sequences having at least 70% or 75%, preferably at least 80% or 85%, more preferably at least 90%, most preferably at least 95%, especially preferably 100% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complementary strand thereof, under the control of a plant expressible promoter.
Furthermore, the invention pertains to a recombinant polynucleic acid, which is a sesquiterpenoid modulating gene (SMG), comprising one or more polynucleic acid sequences, each under control of a plant-expressible promoter, such as, but not limited to those sequences described above or parts thereof, which when expressed in a cell of a plant either induce, increase or decrease the activity of a sesquiterpenoid synthase, such as germacrene A synthase, in that cell.

The present invention also relates to probes and primers derived from the new germacrene A synthase genes that are useful for instance for the isolation of additional germacrene A synthase genes having sequences which differ from SEQ ID NO 1 to 4 by techniques known in the art, such as PCR cloning.
The term "probe" according to the present invention refers to a single-stranded oligonucleotide which is designed to specifically hybridize to any of the germacrene A synthase polynucleic acids of the invention.
The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product which is complementary to the germacrane synthase A gene nucleic acid strand to be copied. Preferably the primer is about 5-50 nucleotides long. The term "target region" of a probe or a primer according to the present invention is a sequence within the germacrene A synthase polynucleic

acid(s) to which the probe or the primer is completely complementary or partially complementary (i.e. with some degree of mismatch). It is to be understood that the complement of said target sequence is also a suitable target sequence in some cases.

"Specific hybridization" of a probe to a target region of the germacrene A synthase polynucleic acid(s) means that the probe forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions this probe does substantially not form a duplex with other regions of the polynucleic acids present in the sample to be analysed.

"Specific hybridization" of a primer to a target region of the germacrene A synthase polynucleic acid(s) means that, during the amplification step, said primer forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions the primer does not form a duplex with other regions of the polynucleic acids present in the sample to be analysed. It is to be understood that "duplex" as used hereby, means a duplex that will lead to specific amplification.

Preferably, the probes of the invention are about 5 to 50 nucleotides long, more preferably from about 10 to 25 nucleotides. Particularly preferred lengths of probes include 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides. The nucleotides as used in the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides containing modified groups which do not essentially alter their hybridization characteristics.

Probe and primer sequences are represented throughout the specification as single stranded DNA oligonucleotides from the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein T is replaced by U).

For designing probes with desired characteristics, the following useful guidelines known to the person skilled in the art can be applied.

The extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, such as degree of complementarity, stability of the probe:target nucleic acid and hybridization conditions including ionic strength, incubation temperature and presence of chemical reagents. Manipulation of one or more of those factors will

determine the exact sensitivity and specificity of a particular probe. The importance and effect of various assay conditions are well known to the person skilled in the art.

Standard hybridization and wash conditions are disclosed in the Examples section. Other conditions are for instance 3X SSC (Sodium Saline Citrate), 20% deionized FA (Formamide) at 50°C. Other solutions (SSPE (Sodium saline phosphate EDTA), TMAC (Tetramethyl ammonium Chloride), etc.) and temperatures can also be used provided that the specificity and sensitivity of the probes is maintained. When needed, slight modifications of the probes in length or in sequence have to be carried out to maintain the specificity and sensitivity required under the given circumstances.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, obtained by introducing into their genome a recombinant polynucleic acid comprising one or more DNA sequences having at least 70% or 75%, preferably at least 80% or 85%, more preferably at least 90%, most preferably at least 95%, especially preferably 100% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complementary strand thereof, each under the control of a plant expressible promoter.

Furthermore, the invention pertains to a recombinant polynucleic acid, which is a sesquiterpenoid modulating gene (SMG), comprising one or more polynucleic acid sequences, each under control of a plant-expressible promoter, such as, but not limited to those sequences described above or parts thereof, which when expressed in a cell of a plant either induce, increase or decrease the activity of a sesquiterpenoid synthase, such as germacrene A synthase in that cell.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, obtained by introducing into their genome a recombinant polynucleic acid comprising one or more DNA sequences having at least 70% or 75%, preferably at least 80% or 85%, more preferably at least 90%, most preferably at least 95%, especially preferably 100% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complementary strand thereof, each under control of a plant expressible-promoter.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, having modified sesquiterpenoid synthase activity, due to the presence in their genome of one or more transgenes which, when expressed inhibit the activity of a sesquiterpenoid synthase, such as a germacrene A synthase, or another enzyme involved in the biosynthesis of sesquiterpenoids in plants. For instance, such enzymes can be enzymes involved further down in the biosynthesis pathway of the sesquiterpene lactone biosynthesis, such as those responsible for the hydroxylation of germacrene A, the formation of the lactone ring and biosynthesis of costunolide (Fransen et al., 1999, Poster abstracts of the '99 BIOTRANS meeting, 26/9-1/10, Taormina, Sicily, Italy, p. 76; De Kraker et al., 2000, Poster presentation at the 10th Symposium ALW discussion group on Secondary Metabolism in Plant and Plant Cell, Feb. 11, Amsterdam, The Netherlands)

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, having modified taste or pathogen resistance due to the presence in their genome of one or more transgenes which, when expressed inhibit the activity of a sesquiterpenoid synthase, such as a germacrene A synthase, or other enzymes involved in the biosynthesis of sesquiterpenoid lactones from germacrene A.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, having modified taste or pathogen resistance due to the presence in their genome of one or more transgenes which, when expressed induce or increase the activity of a sesquiterpenoid synthase, such as a germacrene A synthase, or other enzymes involved in the biosynthesis of sesquiterpenoid lactones from germacrene A.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, having modified sesquiterpene lactone production due to the presence in their genome of one or more transgenes which, when expressed induce or increase the activity of a sesquiterpenoid synthase, such as a germacrene A synthase, or other enzymes involved in the biosynthesis of sesquiterpenoid lactones from germacrene A.

The invention further encompasses a process for modifying taste and/or resistance in a plant, plant organ, tissue or cell comprising introducing one or more recombinant polynucleic acids which induce, increase, decrease or inhibit the expression or activity of a sesquiterpenoid synthase, such as germacrene A synthase, or other enzymes involved in the biosynthesis of sesquiterpenoid lactones from germacrene A.

More particularly, the invention relates to a process for decreasing the bitter taste in a plant, plant organ, tissue or cell, comprising introducing into plant cells or tissues on or more recombinant polynucleic acids comprising a polynucleic acid sequence having at least 70% or 75%, preferably at least 80% or 85%, more preferably at least 90%, most preferably at least 95%, especially preferably 100% similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4 or parts thereof, or the complementary strand thereof, under the control of a plant-expressible promoter, regenerating the transformed plant cells or tissues into plants and obtaining the plants, plant organs, tissues or cells having decreased bitter taste.

Brief description of the drawings

The following detailed description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying figures, incorporated herein by reference, in which:

Figure 1. Enzyme activity of 0.5 mL fractions eluting from a Mono-Q anion exchange column (FPLC).

Figure 2. Radio-GLC traces showing radio-labeled products of enzyme assays on A, fraction 20 (elution volume 10 mL) and B, fraction 26 (elution volume 13 mL) of the Mono-Q eluent shown in Fig. 1. The major peak in both traces represents germacrene A, the minor peaks are rearrangement products of germacrene A.

Figure 3. Radio-GLC analysis of radiolabeled products formed from [^{3}H]-farnesyl diphosphate in assays with protein extracts from transformed *E. coli* BL 21 (DE3) cells (Stratagene). A, FID signal showing an unlabelled authentic standard of germacrene A. B, C, radio-traces showing enzymatic products of protein extracts from BL 21 (DE3) cells transformed with A, the short cDNA and B, the long cDNA. **1,** selina-4, 11-diene **2,3,** α- and ß-selinene **4,** germacrene A. Long and short cDNA refer to the genes encoding the iso-enzymes of germacrene A synthase, of which the cDNA sequences are provided herein.

Figure 4. GC-MS analysis on an HP5-MS column of products formed from farnesyl diphosphate in assays with protein extracts from transformed *E. coli* BL 21 (DE3) cells (Stratagene). A, chromatogram of the negative control (vector without insert); B, chromatograms of B, the short cDNA; C chromatogram of C, the long cDNA; D, an authentic standard of germacrene A. **1,** selina-4,11-diene **2,** ß-selinene **3,** α-selinene **4,** germacrene A. "Long" and "short" cDNA refer to the genes encoding the iso-enzymes of germacrene A synthase, of which the cDNA sequences are provided herein.

Figure 5. Mass spectra of main product peaks **4** from chromatograms in Figure 4; A: the "short" cDNA; B: the "long" cDNA; C: authentic standard of germacrene A.

Figure 6. GC-MS analysis on an enantioselective column (Selected Ion Monitoring-mode) of products formed from farnesyl diphosphate in assays with protein extracts from transformed *E. coli* BL 21 (DE3) cells (Stratagene) with co-injection of an authentic standard of (+)- and (-)-ß-elemene. (A,B) chromatograms of the short cDNA, with A, an injection port temperature of 150°C and B, an injection port temperature of 250°C. C,D, chromatograms of the long cDNA, with C, an injection port temperature of 150°C and D, an injection port temperature of 250°C. **1,** (+)-ß-elemene; **2,** (-)-ß-elemene; **3,** α-selinene; **4,** ß-selinene; **5,** selina-4,11-diene; **6,** germacrene A. "Long" and "short" cDNA refer to the genes encoding the iso-enzymes of germacrene A synthase, of which the cDNA sequences are provided herein.

Figure 7. (a) Northern blot analysis of the expression of the two germacrene A synthase genes in several tissues of chicory; (b) Quantification of Northern blot analysis of the expression of the two germacrene A synthase genes in several tissues of chicory.

Figure 8. Radio-GC analysis of radiolabeled products of incubations of crude extracts of etiolated seedlings of *Cichorium intybus* (A), inuline chicory (B), *Lactuca sativa* (C), radicchio (Chioggia type) (D), radicchio (Treviso type) (E), and endive (F) and *Lactuca sativa* (C) with 3H-labeled farnesyl diphosphate as substrate. Peaks: 1, germacrene A; 2, α/β-selinene; 3, farnesol.

Figure 9. GC-MS spectrum of A, the major sesquiterpene product of an incubation of a crude extract of etiolated seedlings of *Lactuca sativa* with farnesyl diphosphate, and B, of an authentic standard of germacrene A.

Figure 10. (a). GC-MS analysis of the products of an enzyme assay using 50 µM FPP as substrate. A, standard of germacrene A; B, control sample (tobacco transformed with GUS gene); C, D, trangenic tobacco expressing the long germacrene A synthase gene. Injected with an injection port temperature of 150°C (C) or 250°C (D). 1, β-elemene, 2, germacrene A. (b) Sesquiterpene synthase activity of tobacco in vitro regenerants. Wt, wildtype, non-transformed, tobacco plantlets (also regenerated in vitro). 121-17, 8 and 16, transgenic plants containing the GUS gene. E, (putative) transgenic tobacco plants containing the long germacrene A synthase gene. (c) Sesquiterpene synthase activity of tomato Micro-Tom regenerants. C1, C2: transgenic control plants containing a GUS construct. Numbers 2-14: (putative) transgenic tomato plants containing the long germacrene A synthase gene.

Figure 11. Constructs for use in the production of transgenic plants with decreased germacrene A synthase activity. A= gene 1, B=gene 2 (or fragments thereof), A5'= 5'end of gene 1, B5' = 5' end of gene 2, Prom = promoter, T = terminator. Gene 1 and gene 2 refer to the cDNAs encoding the isoenzymes of germacrene A synthase, of which the sequences are provided herein (or fragments thereof) The arrows in the boxes represent the sense and anti-sense orientation of the DNA sequence. The 5' ends

comprise several hundred basepairs including part of the UTR. The promoter is preferably an enhanced 35S promoter, the terminator a nos terminator.

Figure 12. Sesquiterpene synthase activity of chicory in in vitro regenerants. C, transgenic plants containing the GUS gene. A, D and E, plants harboring constructs containing the germacrene A synthase gene(s); A, construct 1; D, construct 4 (Figure 11). E, sense construct of the long germacrene A synthase (Example 7);

Figure 13. Sequence alignment of the two cDNAs (A = "short", SEQ ID NO 3; B = "long", SEQ ID NO 4) encoding germacrene A synthase isolated from chicory. Sequence alignment was done with the ClustalW program.

Figure 14. Sequence alignment of the deduced amino-acid sequences (A= "short", SEQ ID NO 7; B = "long", SEQ ID NO 8) of two iso-enzymes of germacrene A synthase in chicory. Sequence alignment was done with the ClustalW program.

Description of the invention

The term "gene" as used herein refers to any DNA sequence comprising several operably linked DNA fragments such as a promoter and a 5' untranslated region (the 5'UTR), which together form the promoter region, a coding region (which may or may not code for a protein), and an untranslated 3' region (3'UTR) comprising a polyadenylation site. Typically in plant cells, the 5'UTR, the coding region and the 3'UTR (together referred to as the transcribed DNA region) are transcribed into an RNA which, in the case of a protein encoding gene, is translated into the protein. A gene may include additional DNA fragments such as, for example, introns. As used herein, a genetic locus is the position of a given gene in the genome of a plant.

The term "polynucleic acid" refers to DNA or RNA, or amplified versions thereof, or the complement thereof.

The term "chimeric" when referring to a gene or DNA sequence is used to refer to the fact that the gene or DNA sequence comprises at least two functionally relevant DNA fragments (such as promoter, 5'UTR, coding

region, 3'UTR, intron) that are not naturally associated with each other and originate, for example, from different sources. "Foreign" referring to a gene or a DNA sequence with respect to a plant species is used to indicate that the gene or DNA sequence is not naturally found in that plant species. An endogenous plant gene is a gene that is naturally found in the concerned plant species.

As used herein the term "transgene" refers to a recombinant DNA or polynucleic acid molecule that is introduced into the genome of a plant. The term "recombinant DNA or polynucleic acid molecule" is used to exemplify and thus can include an isolated nucleic acid molecule which can be DNA and which can be obtained through recombinant or other procedures. This recombinant DNA molecule usually comprises at least one copy of at least one "gene of interest" (e.g. a recombinant DNA) which is capable of conferring one or more specific characteristics to the transformed plant. A "transgenic plant" refers to a plant comprising a transgene in the genome of all of its cells.

Expression of the transgene is used to indicate that the gene(s) of interest comprised in the transgene is expressed so as to confer on the plant one or more phenotypic traits (e.g. induced, increased or decreased sesquiterpenoid level) that were intended to be conferred by the introduction of the recombinant DNA molecule – the transforming DNA - used during transformation.

The term "sequence identity" with respect to a nucleotide sequence or an amino acid sequence, refers to the number of positions with identical nucleotides divided by the number of nucleotides in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipmann algorithm (Wilbur and Lipmann, *PNAS USA*, **80**:726, 1983) using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data can be conveniently performed using programs of the intelligeneticsTM Suite (Intelligenetics Inc. CA). Sequences which are essentially identical have a sequence identity of at least about 70% or 75%, advantageously at least about 80%, such as at least about 85%, preferably at least about 90%, especially about 95%, such as at least 97%, and especially about 100%. It is clear that when RNA

sequences are said to be essentially identical or identical, or have a degree of sequence identity with DNA sequences, thymidine (T) in the DNA sequence is considered to be equal to uracil (U) in the RNA sequence. Additionally or alternatively, the term "sequence similarity" with respect to a nucleotide or amino acid sequence is intended to indicate a quantitative measure of similarity between two sequences. Sequence similarity as used herein can be measured using the alignment algorithm of the ClustalW program (Thompson et al., *Nucleic Acids Research* **22(22)**: 4673-7680, 1994). Sequences which are essentially similar have a sequence similarity of at least about 70%, advantageously at least about 75% or 80%, such as at least about 85%, preferably at least about 90%, especially about 95%, such as at least 97%, and especially about 100%.

More particularly, the sesquiterpenoid modulating genes as used herein will comprise a DNA sequence which is essentially similar, or, preferentially, essentially identical or identical to one or both of the nucleotide sequences or encodes an amino acid sequence which is essentially similar, or preferentially, essentially identical to one or both of the amino acid sequences corresponding to germacrene A synthase disclosed herein, more specifically in the nucleotide sequence encoding, or the amino-acid sequence corresponding to the "active domain" of the enzyme. The active domain of sesquiterpenoid synthases, such as germacrene A synthase, was determined by Back and Chappell (1996, above) to stretch from about 40 amino acids before to about 140 amino acids behind the conserved DDXXD region.

Reduced or decreased bitterness as used herein refers to a decrease in bitter constituents, i.e., molecules that confer a bitter taste. In the context of the present invention, bitter constituents are sesquiterpenoid molecules that confer a bitter taste on plants, e.g., sesquiterpenoid lactones. These can be determined qualitatively and quantitatively using HPLC (Price et al., 1990, above; Van Beek et al., 1990, above). Alternatively, the activity of enzymes catalyzing the formation of intermediates in the synthesis of the bitter constituents can be used as a measure for bitterness, such as the activity of germacrene A synthase, which catalyses the production of germacrene A, an intermediate in the production of sesquiterpenoid lactones. An "increase" or "decrease" of bitterness or bitter constituents in a transgenic plant or plant

part, as described herein, is determined relative to a non-transgenic plant or plant part.

Resistance as referred to herein relates to a decreased infection state of a plant by certain insects, nematodes, microorganisms or decreased feeding of vertebrate herbivores. In the context of the present invention, resistance will primarily be the result of an increased deterrence to certain organisms, but can also be the result of an increased toxicity of the plant or plant parts to certain organisms, or the increased attraction to predators of the infecting organism. Alternatively, resistance of a plant or plant part can be measured by the presence therein of sesquiterpenoid molecules with deterring activity. An "increase" in resistance of a transgenic plant or plant part, as described herein, is determined relative to the resistance of a comparable non-transgenic plant or plant part.

As used herein, "modulation of sesquiterpenoids" refers to the influencing of the level of one or more sesquiterpenoids in a plant, and can thus refer either to an induction, increase or decrease of production of sesquiterpenoids in the plant. This modulation is preferably achieved, according to the invention, by influencing the level and/or activity of one or more sesquiterpenoid synthases in a plant. Sesquiterpenoid synthases are enzymes that are involved in the biosynthesis of sesquiterpenoids. A "germacrene A synthase" as used herein refers to an enzyme capable of producing germacrene A, preferably as a stable compound which is released from the enzyme without further processing by that enzyme.

The genes which according to this invention, can be used to modulate the level and/or activity of sesquiterpenoid synthases in plants will generally be referred to as "sesquiterpenoid modulating genes" ("SMGs"). These are foreign or endogenous genes comprising one or more DNAs encoding sesquiterpenoid synthases or transgenes derived from genes encoding sesquiterpenoid synthases, more particularly genes encoding germacrene A synthase, or other enzymes involved in the biosynthesis of sesquiterpenoids in plants. More particularly, the DNAs encoding germacrene A synthase, isolated in the context of the present invention are referred to as the "long" and "short" DNAs herein. Modulation of sesquiterpenoid synthase activity is obtained, according to one embodiment of the invention, by influencing

endogenous gene expression in the plant. This is preferably achieved by introducing into the genome of the plant, one or more transgenes which interact with the expression of endogenous genes, by anti-sense RNA, co-suppression or ribozyme suppression.

Alternatively, introduction of one or more DNA sequences encoding a sesquiterpenoid synthase into the plant genome, in a suitable conformation for gene expression (e.g. under control of a plant-expressible promoter), will result in increased or induced expression of the sesquiterpenoid synthase(s) in the plant, and, in the presence of an adequate substrate, in an increase of the corresponding sesquiterpenoid.

Induced, increased or reduced expression of a sesquiterpenoid synthase gene in a transgenic plant or plant cell as compared to a non-transgenic plant or plant cell can be measured by measuring mRNA levels, or where appropriate, the level or activity of the sesquiterpenoid synthase (e.g. ELISA, activity of the enzyme as indicated by the level of sesquiterpenoid or metabolites thereof (such as sesquiterpenoid lactones) formed. Endogenous sesquiterpenoid synthase expression refers to the expression of a protein with sesquiterpenoid synthase activity which is naturally found in the concerned plant, plant part or plant cell.

The biological activity of a sesquiterpenoid synthase can be measured directly in vitro by incubation of a purified or non-purified sample with the substrate of the sesquiterpenoid synthase, preferably labeled, after which the catalytic activity of the sesquiterpenoid synthase can be measured. For example, germacrene A synthase activity in a sample can be measured by incubating a sample allegedly containing the enzyme with (radiolabeled) farnesyl diphosphate as substrate, after which production of germacrene A can be measured by radio-GC analysis or GC-MS analysis (for example as described in the examples herein).

As used herein, the term a "plant-expressible promoter" refers to a promoter that is capable of driving transcription in a plant cell. This includes any promoter of plant origin, including the natural promoter of the transcribed DNA sequence, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e. certain promoters of viral or bacterial origin such as the Cauliflower Mosaic Virus 35S (CaMV35S) or the T-DNA

gene promoters. The term "plant-expressible promoter" includes, but is not restricted to, constitutive, inducible, and tissue-specific promoters.

The present invention is based on the observation that the bitter taste of certain vegetables, for example chicory, is related to the synthesis of sesquiterpene lactones, for example guianolides. As decreasing the bitterness would increase the market value of some of these vegetables, the biosynthesis of the sesquiterpene lactones in chicory was investigated. It was found that germacrene A is the product of a germacrene A synthase from which it is released without being further converted into other sesquiterpenoids by this enzyme. Other enzymes further modify the germacrene A skeleton to produce the variety of sesquiterpene lactones present in chicory. Germacrene A synthase was partially purified and the corresponding genes were isolated. The present invention is further based on the observation that certain sesquiterpene lactones play a role in the resistance of plants against several organisms. Based on these observations the concept was developed that the genes encoding sesquiterpenoid synthases could be used to influence both flavor and resistance in plants.

As the isolated genes encoding monoterpene-, sesquiterpene- and diterpene cyclases display a strong similarity in gene-organization and in amino acid sequence within domains, it is expected that modulation of gene expression, e.g., by anti-sensing or co-suppression, may influence the expression of different genes displaying such similar structure. It should be understood that such similarities can and should be taken into account when designing the transgenes used in the present invention.

In one embodiment of the invention, the production of the bitter constituents in plants is reduced or suppressed, by modulating the level and/or activity of sesquiterpenoid synthase(s) in the plant involved in the production of guianolides and other sesquiterpenoids associated with the bitter taste in vegetables. More particularly, modulation is achieved by suppressing endogenous germacrene A synthase levels by anti-sense RNA, co-suppression or other methods of gene suppression.

In different vegetables, such as chicory, endives, radicchio, lettuce and artichoke, sesquiterpenoid lactones have been shown to be important bitter

constituents. The isolation from chicory of the sesquiterpenoid synthase involved in the biosynthesis of these bitter constituents as well as the genes encoding this enzyme is described herein (example 1 to 5).

According to one aspect of the invention, a decrease in activity of a sesquiterpenoid synthase in a plant or parts of a plant is obtained by introducing into the cells of the plant one or more transgenes which are sesquiterpenoid modulating genes (SMGs), capable of influencing the level of one or more endogenous sesquiterpenoid synthase(s) in the plant. The sesquiterpenoid modulating genes of the present invention comprise a transcribed DNA sequence under the control of, and fused at its 5' end to, the plant-expressible promoter, whereby the resulting RNA, protein or polypeptide, when expressed in cells of the plant, significantly disturb or reduce the level and/or the activity of the endogenous sesquiterpenoid synthase(s). Alternatively, the stable integration of the transgene(s) into the cell in itself results in a decreased expression of the endogenous sesquiterpenoid synthase gene(s).

Thus, in one embodiment of the invention, the sesquiterpenoid modulating gene (SMG) comprises a DNA which encodes an anti-sense RNA which is complimentary to at least part of the sense mRNA of a sesquiterpenoid synthase gene that is naturally transcribed in the cells. More particularly, the SMG comprises a DNA encoding an anti-sense RNA which is the complement of the sense RNA of a germacrene A synthase gene from chicory, most particularly the complement of SEQ ID NO 3 or 4 or a part thereof. Alternatively, the SMG comprises a DNA encoding an anti-sense RNA which has at least 70% or 75%, preferably at least 80% or 85%, particularly at least 90%, more particularly 95%, especially has 100% sequence similarity to the complement of the sense RNA of the sequence of SEQ ID NO 3 or SEQ ID NO 4, or a part thereof. The anti-sense RNA may be complementary to any part of the sense mRNA (corresponding to part or all of an intron, exon, leader sequence etc., coding or non-coding region). Preferably, the anti-sense RNA is complementary to the sense RNA sequence encoding the active domain of the enzyme. More particularly, the anti-sense RNA comprises a sequence of at least 20 nucleotides, preferably 100 nucleotides complementary to the sense RNA encoded by nucleotide

845 to 1390 of SEQ ID NO 3 or to the sense RNA encoded by nucleotide 906 to 1460 of SEQ ID NO 4. Preferably, the SMG does not encode a functional protein, more particularly it does not encode a protein.

Suppression of germacrene A synthase activity may be obtained using one or more SMGs, which can comprise a DNA sequence which encodes an anti-sense RNA which is identical or similar to the complement of the sequence of SEQ ID NO 3 or part thereof, or a DNA sequence which encodes an anti-sense RNA which is identical or similar to the complement of the sequence of SEQ ID NO 4 or part thereof. Alternatively, an SMG may be used which encodes an anti-sense RNA which is at least 60%, preferably at least 65%, most preferably at least 70% similar to the complement of a sequence between nucleotide 845 and nucleotide 1390 of SEQ ID NO 3 and of a sequence between nucleotide 906 and nucleotide 1460 of SEQ ID NO 4.

In another embodiment of the invention, the transcribed DNA sequence of the SMG comprises a DNA that is strongly homologous or similar to an endogenous sesquiterpenoid synthase sequence, so that introduction of the SMG into the genome of the plant causes the endogenous sesquiterpenoid synthase gene to be suppressed (co-suppression). Suppression of expression of the endogenous gene is achieved by introduction of a SMG comprising a strong plant-expressible promoter operably linked to a DNA so that the resulting transcribed RNA is a sense RNA comprising a nucleotide sequence which has at least 75%, preferably at least 80%, particularly at least 85%, more particularly at least 90%, especially at least 95% similarity with, more especially has 100% sequence similarity to the coding or transcribed DNA sequence (sense) of the endogenous sesquiterpenoid synthase gene of which the expression is to be suppressed. Particularly, The SMG comprises a DNA that displays a sequence similarity with the transcribed DNA region of a germacrene A synthase gene or a part thereof. More particularly, it comprises a DNA with a sequence similarity to the transcribed DNA region of SEQ ID NO 3 or SEQ ID NO 4. Particularly, the transcribed region of the SMG does not code for a protein. Preferably, the transcribed DNA region of the SMG does not code for a functional protein. Suppression of germacrene A synthase activity may be obtained using one or more SMGs, which can comprise one or more DNA sequences which is identical or essentially similar the sequence of SEQ ID NO 3 or part thereof,

or identical or essentially similar to the sequence of SEQ ID NO 4 or part thereof. Alternatively, an SMG may be used which has at least 70% or 75%, preferably at least 80%, most preferably at least 90%, especially at least 95% similarity with, more especially has 100% sequence similarity to a sequence between nucleotide 845 and nucleotide 1390 of SEQ ID NO 3 and to a sequence between nucleotide 906 and nucleotide 1460 of SEQ ID NO 4.

Recently, Waterhouse et al. (*PNAS*, **95(23)**: 13959-64, 1998) have described methods and means to make gene silencing in plants more efficient and predictable, by simultaneous expression of both sense and anti-sense constructs in cells of one plant. The sense and anti-sense nucleic acids may be in the same transcriptional unit, so that a single RNA transcript that has self-complementarity is generated upon transcription.

In an analogous way, Hamilton et al. (*The Plant Journal* 15(6): 737-746, 1998) describe improved silencing e.g. of tomato ACC-oxidase gene expression using a sense RNA containing two additional upstream inverted copies of its 5' untranslated region.

WO 98/53083 describes constructs and methods for enhancing the inhibition of a target gene within an organism, involving the insertion into the gene silencing vector of an inverted repeat of all or part of a polynucleotide region within the vector.

In a further embodiment of the invention, an SMG comprises a DNA, which encodes a specific RNA enzyme or ribozyme, capable of highly specific cleavage of an endogenous sesquiterpenoid synthase gene of a plant. Particularly, the ribozyme encoded by the DNA is targeted against a gene encoding germacrene A synthase, most particularly against the mRNA sequence corresponding to the cDNA of SEQ ID NO 3 and/or SEQ ID NO 4.

It is understood that alternative methods can be developed for decreasing the sesquiterpenoid synthase activity in plants or plant parts in order to reduce bitterness, for instance inhibition the activity of the enzyme itself. Thus, the present invention also relates to sesquiterpenoid modulating genes encoding a protein or polypeptide capable of inhibiting the activity of a sesquiterpenoid synthase, more particularly, capable of inhibiting germacrene A synthase activity. Such an SMG can encode, for instance, an antibody or a fragment of an antibody directed against a germacrene A synthase. More particularly, the

antibody fragment will be directed against an epitope of the protein made up of the sequence of SEQ ID NO 7 and/or SEQ ID NO 8,

According to another aspect of the invention, sesquiterpenoid modulating genes (SMG), are used to increase or induce expression of sesquiterpenoid synthase in a plant, so as to increase the level of sesquiterpenoids conferring resistance to the plant or increase the level of sesquiterpenoids that are interesting for other reasons, for example flavor and fragrance compounds derived from germacrene A or sesquiterpene lactones with pharmacological activity. This is achieved by introducing into the genome of a plant one or more SMGs comprising a DNA encoding a protein with sesquiterpenoid synthase activity, under the control of a plant-expressible promoter. More specifically, the SMG comprises a DNA encoding protein with germacrene A synthase activity. For instance, the SMG can comprise a sequence encoding a protein which has at least 70% or 75%, preferably at least 80% or 85%, most preferably 90%, especially at least 95% similarity with, more especially has 100% sequence similarity to SEQ ID NO 7 and/or SEQ ID NO 8, or a part thereof, encoding a functional part of a germacrene A synthase. Plants particularly suited for this embodiment of the invention are plants already producing sesquiterpene lactones such as for example many of the members of the Asteracea, such as species from the genera *Cichorium, Lactuca,* and *Helenium* (in case upregulation of sesquiterpene lactone formation is required), or plants already producing sesquiterpenes such as for example the genera *Carum, Capsicum, Chamomilla, Cichorium, Citrus, Daucus, Gossypium, Juniperus, Lactuca, Tanacetum, Lycopersicon, Nicotiana, Pogostemon, Vetiveria* (in case the production of germacrene A or other terpenoids derived thereof such as nootkatone is desired). If a high production is required, or when a shortage in FDP, the enzyme's substrate, is anticipated, a recombinant DNA encoding a protein or polypeptide with germacrene A synthase activity may be combined with a DNA encoding FDP or a protein with FDP synthase activity.

According to the invention, sesquiterpenoid synthase expression and/or activity in a plant or in plant parts is modulated by introducing one or more sesquiterpenoid modulating genes (SMGs) into the genome of the plant. The SMG(s) comprise(s) a coding region placed under the control of, and fused at

its 5' end to, a plant-expressible promoter. This promoter can be the natural promoter of an endogenous sesquiterpenoid synthase gene, more particularly the promoter of an endogenous germacrene A synthase gene, most particularly the promoter of a gene corresponding to the cDNA of SEQ ID NO 3 or SEQ ID NO 4.

Alternatively, the SMG is placed under control of a constitutive promoter, directing expression in essentially all cells of the plant. More specifically, the constitutive promoter can be, but is not restricted to, one of the following: a 35S promoter (Odell et al., *Nature* **313**:482-493, 1985), a 35S'3 promoter (Hull and Howell, *Virology* **86**:482-493, 1987), the promoter of the nopaline synthase gene ("PNOS") of the Ti-plasmid (Herrera -Estrella, *Nature* **303**:209-213, 1983) or the promoter of the octopine synthase gene ("POCS", De Greve et al., *J. Mol. Appl. Genet.* **1**(6): 499-511, 1982). It is clear that other constitutive promoters can be used to obtain similar effects.

For specific embodiments of this invention, the use of inducible promoters can provide certain advantages. In one embodiment of the invention, modulation of sesquiterpenoid synthase activity is used to increase pest resistance and can be required only upon infection by pests. It has been observed that infestation of peppers by the spider mite induces germacrene A production, possibly as part of a defense mechanism which leads to production of signal molecules which attract spider mite predators (personal communication). Transformation of such a plant with a sesquiterpenoid synthase gene under control of a promoter which initiates gene transcription upon infection of the plant by the spider mite will increase the production of these signal molecules, improving the natural defence mechanism.

In another embodiment of the invention, modulation of sesquiterpenoid synthase activity is required at least in, but possibly only in certain parts of the plant, making it possible to limit modulation to a certain period of culture or developmental stage of the plant. More particularly, it may be desired to decrease the sesquiterpenoid synthase(s) in the plant specifically in those parts of the plant destined for consumption or processing. More specifically, in a preferred embodiment of the invention the bitterness of chicory is decreased, at least in the shoots (eaten as vegetables) and/or roots (used as a source for sugars and/or feed for cattle). The shoots and roots of chicory are grown under dark conditions. Thus, in one aspect of the invention, the

sesquiterpenoid modulating gene(s), is (are) placed under the control of a promoter which directs expression in the cells of the plant under specific dark conditions.

Alternatively, for other bitter tasting plants of which the leaves are used as food crop, such as, but not limited to, the lettuce (*Lactuca sativa*), light-inducible promoters can be used. Examples of inducible promoters are the dark regulated PRB-1b protein promoter described by Sessa et al. (*Plant Mol. Biol.*, **28**(3): 537-547, 1995) and the dark and light regulated chlorophyll A/B binding protein promoters, described by Cashmore (*Proc. Natl. Acad. Sci.* **81**:2960-2964, 1984) and by Simpson, et al. (*EMBO J.* **4**:2723-2729, 1985) and in US patent n° 5,656,496.

Similarly, an inducible increase in sesquiterpenoid production can be of interest to protect plants from insects, fungi, nematodes or vertebrate herbivores by placing the SMG(s) under the control of an insect- fungus-, nematode-, or wounding-inducible promoter.

For specific embodiments of this invention, the use of tissue-specific promoters can provide certain advantages. More particularly, reduction of bitterness in plants will mainly be of value for parts of the plants destined for consumption or processing. Thus, in specific embodiments of the invention, the SMG(s) is (are) placed under the control of a promoter directing expression in specific plant tissues, such as roots or leaves. For instance, in chicory, reduction of the activity of sesquiterpenoid synthase, more particularly germacrene A synthase, is directed at the shoots (eaten as vegetables) and/or the roots (used as a source for sugars).

Similarly, in plants where certain parts of the plants are particularly susceptible to the damage of insects, microorganisms, nematodes or vertebrate herbivores, tissue-specific increase in sesquiterpenoid production can be of interest. For instance, to protect plants from infection by aphids, an increase in sesquiterpenoid synthase production is directed to the phloem or the chlorophyll-producing plant parts. More particularly, the SMG(s) is (are) placed under the control of a phloem-specific promoter (such as the rolC promoter of Agrobacterium) or the promoter of the gene encoding the small subunit of Rubisco. Alternatively, to protect plants from infection by root pathogens, for example fungi or nematodes, the increase in sesquiterpenoid synthase production is directed to the roots. More particularly, the SMG(s)

is(are) placed under the control of a root-specific promoter (such as described by Keller et al., *Genes Devel.* 3: 1639-1646, 1989).

The sesquiterpenoid modulating gene(s) may include further regulatory or other sequences from other plant or bacterial genes, such as leader sequences (e.g. the cab22 leader from Petunia), 3'transcription termination and polyadenylation signals (e.g. from the octopine synthase gene or the nopaline synthase gene), plant translation initiation consensus sequences, introns etc, which is or are operably linked to the SMG.

The recombinant DNA comprising one or more SMGs may be accompanied by a chimeric marker gene. The chimeric marker gene can comprise a marker DNA that is operably linked at its 5' end to a plant-expressible promoter, preferably a constitutive promoter, such as the CaMV 35S promoter, or a light inducible promoter such as the promoter of the gene encoding the small subunit of Rubisco; and operably linked at its 3' end to suitable plant transcription 3' end formation and polyadenylation signals. It is expected that the choice of the marker DNA is not critical, and any suitable marker DNA can be used. For example, a marker DNA can encode a protein that provides a distinguishable color to the transformed plant cell, such as the A1 gene (Meyer et al., *Nature* **330**: 677, 1987), can provide herbicide resistance to the transformed plant cell, such as the *bar* gene, encoding resistance to phosphinothricin (EP 0,242,246), or can provide antibiotic resistance to the transformed cells, such as the *aac(6')* gene, encoding resistance to gentamycin (WO94/01560).

The cell of a plant is preferably transformed in accordance with this invention, using a vector that is a disarmed Ti-plasmid containing the transgene and carried by *Agrobacterium*. This transformation can be carried out using the procedures described, for example, in European patent publications 0,116,718 and 0,270,822. Protocols describing *Agrobacterium*-mediated transformation of lettuce, chicory and tobacco are described in Michelmore, R. et al. (*Plant Cell Reports* **6**, 439-442, 1987), Hohn and Ohlrogge, (*Plant Physiology* **97**, 460-462, 1991) and Frulleux et al. (*Plant Cell, Tissue and Organ Culture* **50**, 107-112, 1997). Preferred Ti-plasmid vectors contain the transgene sequence between the border sequences, or at least located to

the left of the right border sequence, of the T-DNA of the Ti-plasmid. Where advantageous, plants are preferably transformed with auxotrophic *Agrobacterium* strains as described in European Patent Application 9711465.3). Of course other methods can be used to transform the plant cell, such as direct gene transfer (as described, for example in EP 0,223,247), pollen-mediated transformation (as described, for example in EP 0,270,356, WO85/01856), in vitro protoplast transformation (as described for example in US patent 4,684,611), plant RNA virus-mediated transformation (as described, for example in European patent publication 0,067,553 and US patent 4,407,956) and liposome mediated transformation (as described, for example, in US patent 4,536,475).

Although it is clear that the invention can be applied essentially to all plant species and varieties, the invention will be especially suited for those plants for which a decrease in bitter constituents or an increased resistance would result in an enhanced commercial value. The obtained transformed plants can be used in a conventional breeding scheme to produce more transformed plants with the same characteristic or to introduce the modified sesquiterpenoid synthase activity characteristic of the invention in other varieties of the same or related plant species. Seeds obtained from the transformed plants contain the transgene of the invention as a stable genomic insert.

The following Examples describe the isolation of a novel sesquiterpenoid synthase gene, the germacrene A synthase gene from *Cichorium intybus* and the use of this sequence or parts thereof in the manipulation of germacrene A synthase activity in plants. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd. (UK) and Blackwell Scientific Publications, UK.

Throughout the Description and Examples reference is made to the following sequences:

SEQ ID NO 1: PCR fragment 1 (short)
SEQ ID NO 2: PCR fragment 2 (long)
SEQ ID NO 3: cDNA 1 encoding C. intybus germacrene A synthase (short)
SEQ ID NO 4: cDNA 2 encoding C. intybus germacrene A synthase (long)
SEQ ID NO 5: amino acid sequence encoded by PCR fragment of SEQ ID NO 1
SEQ ID NO 6: amino acid sequence encoded by PCR fragment of SEQ ID NO 2
SEQ ID NO 7: amino acid sequence encoded by cDNA of SEQ ID NO 3
SEQ ID NO 8: amino acid sequence encoded by cDNA of SEQ ID NO 4
SEQ ID NO 9: primer
SEQ ID NO 10: primer
SEQ ID NO 11: primer
SEQ ID NO 12: primer
SEQ ID NO 13: primer
SEQ ID NO 14: primer

Example 1: Partial purification from chicory of two proteins catalyzing the formation of germacrene A from FDP

Chicory chicons were cut into small pieces, frozen in liquid nitrogen and ground to a fine powder using a cooled mortar and pestle. One gram of this powder was homogenized in 10 mL buffer containing 25 mM Mopso (pH 7.0), 20% (v/v) glycerol, 25 mM sodium ascorbate, 25 mM $NaHSO_3$, 10 mM $MgCl_2$ and 5 mM DTT (buffer A), slurried with 0.5 g polyvinylpolypyrrolidone (PVPP) and a spatula tip of purified sea sand. To the homogenate 0.5 grams of polystyrene resin (Amberlite XAD-4, Serva) were added and the slurry was stirred carefully for 10 min and then filtered through cheesecloth. The filtrate was centrifuged at 20,000*g* for 20 min (pellet discarded), and then at 100,000*g* for 90 min. The 100,000*g* supernatant was loaded on a 10 x 2.5 cm column of Q-sepharose (Pharmacia Biotech) previously equilibrated with buffer containing 15 mM Mopso (pH 7.0), 10% (v/v) glycerol, 10 mM $MgCl_2$ and 2 mM DTT (buffer B). The column was washed with the equilibration buffer and eluted with a 0-2.0 M KCl gradient. For determination of enzyme

activities, 20 µL of the 2.0-ml fractions were diluted 5-fold in an Eppendorf tube with buffer B and 20 µM [^{3}H]FDP was added. The reaction mixture was overlaid with 1 mL of hexane to trap volatile products and the contents mixed. After incubation for 30 min at 30°C, the vials were mixed, and centrifuged to separate phases. A portion of the hexane phase (750 µL) was transferred to a new Eppendorf tube containing 40 mg of silica gel, and, after mixing and centrifugation, 500 µL of the hexane layer was removed for liquid scintillation counting in 4.5 ml of Ultima Gold cocktail (Packard).

The combined active fractions were desalted to buffer B, and 1.0 mL of this enzyme preparation was applied to a Mono-Q FPLC column (HR5/5, Pharmacia Biotech), previously equilibrated with buffer B containing 0.1% Tween-20. The column was eluted with a gradient of 0-600 mM KCl in the same buffer. The activity, which eluted as one peak from the Q-sepharose column, was now separated into two activity peaks (activity assessed as described above) (Fig. 1). For determination of product identity, 0.5 mL of the two most active fractions were diluted 2-fold with buffer B and 20 µM [^{3}H]-FDP were added. After the addition of a 1-mL redistilled pentane overlay, the tubes were carefully mixed and incubated for 1 h at 30°C. Following the assay, the tubes were mixed, the organic layer was removed and passed over a short column of aluminum oxide overlaid with anhydrous $MgSO_4$. The assay was extracted with another 1 mL of diethyl ether, which was also passed over the aluminum oxide column, and the column washed with 1.5 mL of diethyl ether. Before radio-GLC-analysis the extract was carefully concentrated under a stream of N_2.

Radio-GLC was performed on a Carlo-Erba 4160 Series gas chromatograph equipped with a RAGA-90 radioactivity detector (Raytest, Straubenhardt, Germany). Sample components eluting from the column were quantitatively reduced before radioactivity measurement by passage through a conversion reactor filled with platinum chips at 800°C. Samples of 1 µL were injected in the cold on-column mode. The column was a fused silica capillary (30 m x 0.32 mm i.d.) coated with a film of 0.25 µm of polyethylene glycol (EconoCap EC-WAX, Alltech Associates) and operated with a He-flow of 1.2 mL min^{-1}. The oven temperature was programmed to 70°C for 5 min, followed by a ramp of 5° min^{-1} to 210°C and a final time of 5 min. About 20% of the column effluent was split with an adjustable splitter to an FID (temperature 270°C). The remainder was directed to the conversion reactor and radio detector. H_2

was added prior to the reactor at 3 mL min^{-1}, and CH_4 as a quench gas prior to the radioactivity detector (5 mL counting tube) to give a total flow of 36 mL min^{-1}. Both fractions showed exactly the same radiolabeled products, with the major peak belonging to germacrene A (Fig. 2), proving that chicory contains two distinct proteins both catalyzing the formation of germacrene A from FDP. The minor peaks preceding the germacrene A peak, belong to rearrangement products of germacrene A, *viz.* α/ß-selinene, and selina-4,11-diene (De Kraker et al., 1998, above).

Example 2: Isolation of the genes encoding germacrene A synthase

a) Isolation of mRNA. Total RNA was isolated from chicory chicons using the purescript RNA isolation kit (Biozym). DNase I (Deoxyribonuclease I, RNase free) was used to remove DNA from the RNA isolate. The DNase I was removed with a phenol/chloroform extraction after which the RNA was precipitated (ethanol precipitation with NaAc). Poly(A) + RNA was extracted from 20 µg of total RNA using 2 µg poly-d(T)25V oligonucleotides coupled to 1 mg paramagnetic beads (Dynal A.S.). The poly(A) + RNA was resuspended in 20 µl H_2O.

b) cDNA synthesis. The reverse transcription reaction was carried out in a 50 µl reaction containing 10 µl poly (A) + mixture, 0.3 µg oligo $(dT)_{25}V$, 1 mM each dATP, dTTP, dCTP and dGTP, 50 mM Tris-HCl pH=8.3, 80 mM KCl, 10 mM MgCl2 and catalyzed with 12 U AMV reverse transcriptase (Pharmacia). After an incubation for 2h at 42°C the reaction was stopped and the cDNA purified with the Wizard PCR Preps DNA purification system (Promega). The cDNA was resuspended in 50 µl H_2O.

c) PCR-based probe generation.

Based on comparison of sequences of terpenoid synthases, two degenerated primers were designed for two conserved regions (Yuba et al., *Archives of Biochemistry and Biophysics*, **332:** 280-287,1996).

sense primer (primer A):

5'-TTY CAR GAY GAR AAY GGI AAR TTY AAR GA-3' (SEQ ID NO 9)

wherein Y=C/T and R=G/A

anti-sense primer (primer B):

5'-CC RTA IGC RTC RAA IGT RTC RTC -3' (SEQ ID NO 10)

wherein Y=C/T and R=G/A

PCR was performed in a total volume of 50 µl containing 0.5 µM of each of the two primers, 0.2 mM dNTP, 1 U Super Taq polymerase / 1x PCR buffer (HT Biotechnology LTD, Cambridge, England) and 10 µl cDNA. The reaction mixture was incubated in a thermocycler (Robocycler, Stratagene) with 1 min denaturation at 94°C, 1.5 min annealing at 42°C and 1 min elongation at

72°C during 40 cycles. Agarose gel electrophoresis revealed a single specific PCR product of approximately 550 bp. The PCR product was purified using the Wizard PCR Preps DNA purification system (Promega) and subcloned using the pGEMT system. *E. coli* JM101 was transformed with this construct. 12 individual transformants were sequenced which resulted in two different sequences (SEQ ID NO 1 and SEQ ID NO 2, the deduced amino acid sequences (primers included) are shown in SEQ ID NO 5 and 6, respectively).

d) cDNA library construction and screening.

A cDNA library was constructed using the UniZap XR custom cDNA library service (Stratagene). For library screening 200 ng of both PCR amplified probes were gel-purified, randomly labeled with [α-^{32}p]dCTP, according to manufacturer's recommendation (Ready-To-Go DNA labeling beads (-dCTP), Pharmacia) and used to screen replica filters of 10^4 plaques of the cDNA library plated on *E. coli* XL1-Blue MRF' (Stratagene). The plaque lifting and hybridization were carried out according to standard protocols. Positive clones were isolated using a second and third round of hybridization. *In vivo* excision of the pBluescript phagemid from the Uni-Zap vector was performed according to manufacturer's instructions (Stratagene). Two groups of positive clones were obtained which could be distinguished using restriction enzymes and specific PCR primers. The cDNA sequences of two representatives of the two groups are shown in SEQ ID NO 3 and 4. These were named the "short" and "long" germacrene A synthase cDNAs (also referred to as gene 1 and gene 2 or A and B). The deduced amino acid sequences are shown in SEQ ID NOs 7 and 8, respectively. Nucleotide and amino acid sequence alignments are shown in Figures 13 and 14 respectively.

Example 3: Expression of the isolated genes in *E. coli*

For functional expression, the cDNA clones were subcloned in frame into the expression vector pET 11d (Stratagene). To introduce suitable restriction sites for subcloning, gene A was amplified by PCR using the following sense and anti-sense primers:

sense primer:

5'-CAA TCC GAA CCA TGG CTC TCG TT-3' (SEQ ID NO 11)

(introducing an *Nco*I site at the start codon **ATG**)

anti-sense primer:
5'- CAC CAA ATG GAT CCA AAT TCG C-3' (SEQ ID NO 12)
(introducing a *BamH*I site behind the stop codon **TGA**).

Gene B was amplified by PCR using the following sense and anti-sense primers:

sense primer:
5'-CCT TCA AGC CAT GGC AGC AGT TG-3' (SEQ ID NO 13)
(introducing an *Nco*I site at the start codon **ATG**)

anti-sense primer:
5'-TTG TAA TAG GAT CCA CTA TAG G-3' (SEQ ID NO 14)
(introducing a *BamH*I site behind the stop codon **TGA**)

The PCR reaction was performed under standard conditions. After digestion with *BamH*I and *Nco*I, the PCR product and the expression vector pET 11d were gel purified and ligated.
The two constructs and pET 11d without an insert (as negative control) were transformed to *E. coli* BL 21 (DE3) (Stratagene), and grown overnight on LB agar plates supplemented with ampicillin at 37°C. Cultures of 50 ml LB medium supplemented with ampicillin (100 µg/ml) and 0.25 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) were occulated with these over night cultures to A_{600} = 0.5 and grown for 3 h at 27°C. The cells were harvested by centrifugation during 8 minutes at 2000 g and resuspended in 1.2 ml buffer B containing 1 mM sodium ascorbate (buffer C). The resuspended cells were sonicated on ice during 4 min (5 sec on, 30 sec off), centrifuged for 5 minutes at 4°C (14.000 rpm) and the supernatant used for assays.

Example 4: Verification of product identity of cDNAs expressed in *E. coli*

For determination of product identity, 20 µM [^{3}H]-FDP was added to 0.5 mL of the enzyme preparations diluted 1:1 with buffer C containing 0.1% tween-20. After the addition of a 1-mL redistilled pentane overlay, the tubes were

carefully mixed and incubated for 1 h at 30°C. Following the assay, the tubes were mixed, the organic layer was removed and passed over a short column of aluminum oxide overlaid with anhydrous $MgSO_4$. The assay was re-extracted with 1 mL of pentane:diethyl ether (80:20), which was also passed over the aluminum oxide column, and the column washed with 1.5 mL of pentane:diethyl ether (80:20). The extract was analyzed using radio-GLC as described above (Example 1) and using GC-MS as described below. Radio-GLC analysis showed that both cDNAs formed functionally active proteins catalyzing the formation of three or more radiolabeled sesquiterpenes from [^{3}H]-FDP (Fig. 3). The negative control (vector without insert) produced no radioactivity. The samples were also analyzed by GC-MS using a HP 5890 series II gas chromatograph equipped with an HP-INNOWax column (30 m x 0.25 mm i.d., 0.25 µm df) and HP 5972A Mass Selective Detector (Hewlett-Packard). The oven was programmed at an initial temperature of 70°C for 1 min, with a ramp of 5°C min^{-1} to 210°C and final time of 5 min. The injection port (splitless mode), interface and MS source temperatures were 150, 290 and 180°C, respectively, and the He inlet pressure was controlled by electronic pressure control to achieve a constant column flow of 1.0 mL/min. Ionization potential was set at 70 eV, and scanning was performed from 30-250 amu. The negative control produced no sesquiterpenes (Fig 4(a)), whereas in assays with the expression products of both the short (Fig. 4(b)) and the long cDNA (Fig. 4(c)) four different sesquiterpenes could be detected: selina-4,11-diene (**1**), ß-selinene (**2**), α-selinene (**3**), and germacrene A (**4**) as major product. The identity of the latter was confirmed by analysis of an authentic standard of germacrene A (courtesy of Dr W.A. König) (Fig. 4(d)), and comparison of the mass spectra with the authentic standard (Fig. 5). The other three products **1,2,3** are rearrangement products of germacrene A, i.e. they are not produced enzymatically (Teisseire, P.J., *Chemistry of fragrant substances*, VCH Publishers Inc., USA, 1994; De Kraker, J-W. et al., *Plant Physiology* **117:** 1381-1392, 1998).

The chirality of the germacrene A produced by the two genes was assessed by GC-MS using the fact that the high-temperature-induced Cope-rearrangement of germacrene A to ß-elemene occurs with retention of stereochemical configuration at C7 (De Kraker et al, 1998, above). GC-MS analysis was carried out essentially as described above, but the GC was equipped with a 25 m (0.25 mm i.d.) heptakis (6-O-TBDMS-2,3-di-O-methyl)-

β-cyclodextrin (50% in OV17) column that is able to separate the enantiomers of ß-elemene (König et al., *J. High Resolut. Chromatogr.* **17:** 315-320, 1994). The oven temperature was programmed to 45°C for 4 min followed by a ramp of 2°C min^{-1} to 170°C, and spectra were recorded in scan or Selected Ion Monitoring mode (m/z 121, 147 and 189). The injection port temperature was either 150°C (no Cope-rearrangement of germacrene A) or 250°C (Cope-rearrangement of germacrene A to ß-elemene). A standard of (+)- and (-)-ß-elemene was co-injected with the germacrene A produced by the two clones. Figs 6A and C show the chromatograms of the short and the long clone, respectively, with co-injection of (+)- and (-)-ß-elemene at an injection port temperature of 150°C. Germacrene A (**6**) is by far the major product, with small amounts of the proton-induced rearrangement products α-selinene (**3**), ß-selinene (**4**) and selina-4,11-diene (**5**). The two enantiomers of ß-elemene are separated: (+)-ß-elemene (**1**) and (-)-ß-elemene (**2**). When the injection port temperature is increased only the (-)-enantiomer of ß-elemene is formed from the germacrene A of both clones. This was demonstrated using scan measurements also without co-injection with the ß-elemene standard (data not shown). Upon co-injection with the (+)- and (-)-ß-elemene standard and using a high injection port temperature, (only) the (-)-ß-elemene peak areas increase for both clones (Figs 6B,D). This proves that the germacrene A produced by both clones is exclusively rearranged to (-)-ß-elemene, implying that both clones produce exclusively (+)-germacrene A (De Kraker et al, 1998, above). Thus it was concluded that the two sequences encode iso-enzymes of germacrene A synthase (also referred to as "long" and "short" or A and B).

Example 5: Expression pattern of the two genes in chicory

The expression pattern of the two germacrene A synthase genes was investigated in Chicory by Northern blot analysis. RNA from different tissues was isolated using the Wizard system (SV Total RNA Isolation System, Promega) according to the procedure recommended by the manufacturer. Of each sample 2 µg of total RNA, treated with DMSO glyoxal, was separated on a 1% gel and blotted onto Hybond-N+ nylon membrane using 7.5 mM NaOH as described by Sambrook et al (Molecular cloning, second edition, 1989, 7.40-7.50). To fix the RNA, the membrane was exposed to Ultraviolet

light (254 nm). Prehybridization and hybridization itself were carried out according to the procedures recommended by the manufacturer (Amersham) in a solution containing 5 x SSPE, 5 x Denhardt's solution, 0.5 % SDS and 0.1 mg/ml herring sperm DNA. Subsequently, the blots were washed at 65°C with 0.1 x SSPE and 0.1 % SDS and exposed to a Fuji Photo Film. The pattern on the film was analyzed using the BAS 2000 Image Plate Scanner.
The probe used in the hybridization step was generated using the Ready to go system according to the procedure recommended by the manufacturer (Pharmacia). As templates for the probes (gel-) purified PCR fragments of the genes to be analyzed were used.

There were marked differences in the expression of the two genes in the different tissues tested (Figure 7(a),(b)). The short gene was expressed particularly in the root tissues (about equally in the outer and core tissue) and in the seedlings, but hardly in the chicon and the green leaves. The long gene was expressed strongest in the root outer tissue, and much less in the root inner tissue. It was expressed at a simmilar level in chicon tissue and seedlings and much lower in green leaves.
These results suggest that in order to reduce bitterness in the chicon tissue of Chicory inhibition of the long gene could be sufficient.

Example 6: Germacrene A synthase activity in other plants

In addition to chicory, several other crops belonging to the Compositae have been shown to contain bitter sesquiterpene lactones (Price et al., 1990, above). It was therefor investigated whether germacrene A synthase activity could be detected in other lettuce, endive and chicory varieties containing these lactones. Etiolated seedlings of seven different varieties were grown for a period of 8 days at 20°C in darkness after which an enzyme extract was made of the seedlings. These extracts were incubated with radiolabeled farnesyl diphosphate, the ubiquitous precursor of sesquiterpenes. Radio-GC analysis was performed on these samples.
All seven samples displayed germacrene A synthase activity (peak 1, Figure 8). The identity of peak 1 was confirmed using GC-MS, of which one example is shown in Figure 9. The fronting of peak 1 and 2 represents selina-4,11-diene and α- and β-selinene, acid-induced rearrangement products of germacrene A (De Kraker et al., 1998, above) Peak 3 represents farnesol,

produced from FDP by non-specific phosphohydrolase activity. These results strongly suggest that also in other lettuce, endive and chicory varieties, sesquiterpene lactone biosynthesis proceeds via the central intermediate germacrene A.

Example 7: Transformation of tobacco and tomato with the germacrene A synthase gene(s) to obtain (increased) germacrene A or sesquiterpene lactone formation

To induce or upregulate the production of sesquiterpene lactones or the production of germacrene A in transgenic plants, plants were transformed using plasmids comprising a sesquiterpenoid synthase modulating gene comprising a sequence encoding protein or polypeptide having germacrene A synthase activity, placed under the control of a suitable plant-expressible promoter, such as a constitutive promoter.

Tobacco transformation. For the transformation of Nicotiana tabacum (Petit Havana SR1) leaf disks were immersed in an antibiotic-free suspension of the A. tumefaciens strain LBA4404 transformed with pBI.E(LB medium, OD600 = 0.5). The pBI.E construct is a derivative of pBI121 in which the gus gene was replaced by the long germacrene A gene. The infected leaf disks were incubated on co-cultivation medium (MS, 20 g/l sucrose, 1 mg/l NAA, 0.2 mg/l BAP, 6 g/l agar pH 5.8) for 3 days at 25°C under continuous dimmed light.To select and regenerate transformed tissue the leafdisks were transferred several times to selection medium (MS, 20 g/l sucrose, 1 mg/l zeatin, 0.1 mg/l NAA, 150 mg/l cefotaxine, 150 mg/l vancomycin, 100 mg/l kanamycin, 6 g/l agar, pH 5.8) at intervals of 7-14 days and incubated at 25°C under continuous dimmed light. The regenerated shoots were maintained on MS media supplemented with 20 g/l sucrose and 100 mg/l kanamycin.

Transgenic plants were screened for the activity of the introduced gene by examining sesquiterpene synthase activity in enzyme extracts. Hereto, 100 mg of tissue, ground in liq N2, was extracted in a 2-ml Eppendorf vial, using a plastic probe to further homogenize the tissue, in 1.0 ml of extraction buffer containing containing 50 mM Mopso (pH 6.8), 20% (v/v) glycerol, 50 mM sodium ascorbate, 50 mM NaHSO3, 1% PVP-40, 10 mM MgCl2 and 5 mM DTT. After extraction, the samples were centrifuged for 20 min at 20,000g. at

4 °C 10 µL of the supernatant were diluted 10 fold with assay buffer containing 15 mM Mopso (pH 7.0), 10% (v/v) glycerol, 10 mM MgCl2, 1 mM sodium ascorbate, 2 mM DTT and 6 mM sodium orthovanadate (an inhibitor of phosphohydrolase activity). After addition of 15 µM 3H-FPP (at 65 Ci mol-1), the reaction mixture was overlaid with 1 mL of hexane to trap volatile products and the contents carefully mixed. After incubation for 30 min at 30°C, the vials were vigorously mixed, and centrifuged (2 min 15.000g 4°C) to separate phases. A 750-µL portion of the hexane phase was transferred to a second Eppendorf vial containing approx. 20 mg silica to bind sesquiterpene alcohols produced by any remaining phosphohydrolase activity, vigorously mixed and centrifuged. (10 min 15.000g 4°C) 500 µL of hexane were removed from these vials for liquid scintillation counting in 4.0 ml of Ultima Gold cocktail (Packard). All assays were performed in duplicate.

The wildtype and GUS-construct controls all exhibited a similar sesquiterpene synthase activity (Figure 10 (b)), which is due to the presence of endogenous tobacco sesquiterpene synthases, such as for example the tobacco epi-aristolochene synthase (Facchini and Chappell, 1992). The transformants E24.4, 26, 12.2, 24.3, 26.2 and 25.3 exhibited a significantly higher sesquiterpene synthase activity when compared with the 6 controls showing that transformation with the germacrene A synthase resulted in increased sesquiterpene synthase activity (Figure 10 (c)). Subsequently, the sesquiterpene synthase activity of a number of transformants was assessed on a larger scale. Hereto, 500 mg of ground tissue was homogenized in 4 ml of the extraction buffer described above, and centrifuged for 20 min at 20,000g. The supernatant was desalted to the assay buffer described above. After addition of 50 µM unlabelled FPP to 1 ml of this desalted extract, the reaction mixture was overlaid with 1 ml of redistilled pentane and incubated for 60 min at 30°C. The assays were worked up, and analyzed using GC-MS as described under Example 6. GC-MS analysis showed the presence of several sesquiterpenes in both control and putative transgenic plants. However, transgenic regenerant E12.2 showed a large peak, which was not present in the control samples, with the mass spectrum and retention time of the authentic germacrene A standard (Figure 10(a)). The fact that high injection port temperature causes this peak to shift to a lower retention time

(of β -elemene) confirms that indeed the germacrene A synthase gene was functionally expressed in tobacco.

Tomato transformation. A tobacco (Nicotiana bentiana) cell suspension culture (TCSC) in a liquid NIBE medium (4.4g/l MS salts with B5 vitamins, 3% sucrose, 0.2mg/l BAP, 0.2mg/l 2,4-D NAA, pH 5.8) was set up and used for feederlayers. The cultures were grown for at least 2 weeks on a shaker at 25°C.

A feederlayer consists of 2 ML of a tobacco cell suspension culture incubated on NIBE plates (NIBE medium, 0.8% purified agar (Oxoid), pH 6.0). The feederlayer was set to grow overnight, covered with 4 folds of filter paper, in a culture room (25°C) under low light conditions. The agrobacterium strain harbouring the germacrene A construct were streaked out on LB/RIF/KAN plates (10g/l Bacto tryptone, 5g/l Bacto yeast extract, 10g/l NaCl, 15g/l Bacto agar, pH 7.0, 100mg/l riframycin, 50mg/l kanamycin.

The plates were set to grow for three days at 28°C under dark conditions. A single colony was inoculated in YEP-selection medium (10g/l yeast extract, 10g/l pepton, 5g/l NaCl, pH 7.0, 0.1M glucose, 50mg/l kanamycin, 50mg/l riframycin, 100μM acetosyringone) and set to shake overnight at 28°C.The selection culture was spin down (2500 rpm 15 minutes) and re-suspended in MS20 (4.4g/l MS salts including vitamins, 2% sucrose, pH 5.8).

This Agrobacterium suspension, with an OD600 of 0.2, was used to immerse the explants. The tomato cultivar 'Micro-Tom' (Lycopersicon flavour) was used (Scott and Harbaugh, 1989). The plants were grown from seeds provided by a seed company (Beekenkamp seed, Holland). Micro-Tom seeds were first sterilised. A rinse in 70% ethanol followed by a two hour bleaching in 1.5% HClO4. After bleaching, the seeds were quickly rinsed in water twice and then washed in water for ten and sixty minutes. After sterilisation, seeds were sowed in pots, containing 80ml vermiculite and 70ml of germination medium containing 4.4g/l MS salts with vitamins and 0.5% sucrose (pH 5.8).

After 7 days of growth in a culture room (25°C), covered with 2 folds of filter paper, the cotyledons were cut under water near the petiole and the tip with a rolling action of the scalpel, to minimize damage. The explants were placed on their backs on filter paper on feederlayers to incubate overnight in the culture room (25°C), covered with 4 folds of filter paper, under low light conditions. After incubation, the explants were immersed in the

The down-regulation of genes in plants is achieved in different ways, e.g. through sense and anti-sense inactivation. In order to inhibit the activity of endogenous germacrene A synthases to obtain plant species with decreased production of germacrene A or products derived thereof, for example sesquiterpene lactones, plant species such as chicory and lettuce (and other sesquiterpene lactone producing species) are transformed by the techniques described above using plasmids containing genes coding for the germacrene A synthase(s) in either sense (to obtain co-suppression) or anti-sense orientation.

In order to test the impact of both genes on the production of sesquiterpenoids and the bitter taste of Chicory plants, constructs were made to inhibit either one or both germacrene A synthase genes together. Based on the expression pattern of the genes (Example 5) it was expected that for reduction of bitterness in the chicon, inhibition of the long gene alone could be sufficient.

The coding regions of the two genes encoding isoenzymes of germacrene A synthase of the present invention have a sequence similarity of 67%. Within the region encoding what is believed to be the "active domain" of the enzyme (stretching from about 40 amino acids before to about 140 amino acids behind the conserved DDXXD sequence) the sequence similarity between the two genes is 82.4%. Therefore, constructs are developed which comprise more than one DNA sequence whereby one DNA sequence comprises a DNA encoding an RNA with a sequence similarity to all or part of the sequence of SEQ ID NO 3, or the complementary strand thereof and the other encodes an RNA sequence with a sequence similarity to all or part of the sequence of SEQ ID NO 4, or the complementary strand thereof. The nucleic acid molecule used preferably comprises two sequences whereby the first sequence is identical to the complement of the second sequence, possibly separated by a spacer sequence, so as to form inverted repeats. The DNA sequences are placed under the control of adequate promoters, such as the 35S promoter, and terminator sequences and are introduced into chicory by Agrobacterium mediated transformation.

Examples of the DNA sequences comprise:

Agrobacterium suspension for 20 minutes. After immersion, the explants were placed back on feederlayers for co-cultivation, following a rinse in a solution containing 400mg/l carbenicillin and 100mg/l tricarcillin. The explants were placed in callus inducing medium (4.4g/l MS salts with Nitsch vitamins, 3% sucrose, 0.8% purified agar (Oxoid), pH 6.0, 2mg/l zeatin, 400mg/l carbenicillin, 100mg/l tricarcillin, 100mg/l kanamycin). The plates were covered with 2 folds of filter paper and set to grow in a culture room (25°C) under low light conditions for 3 weeks. Formed callus was transferred to shoot inducing medium (as callus inducing medium, but with 1mg/l zeatin, 200mg/l carbenicillin, 50mg/l tricarcillin).

These plates were set to grow under the same conditions as the callus-inducing plates. Shoots formed were transferred to rooting medium in pots (4.4g/l MS salts with Nitsch vitamins, 3% sucrose, 0.5% agargel (Sigma), pH 6.0, 0.25mg/l IBA, 50mg/l kanamycin, 400mg/l carbenicillin. The growing conditions remained the same. Fully-grown plants were subsequently transferred to the greenhouse.

Sesquiterpene synthase activity was tested as described above for tobacco, but with 10 µM 3H-FPP as substrate. A number of transformants were found to have significantly higher (up to 4 to 5-fold) sesquiterpene synthase activity, as a result of the transformation with the germacrene A synthase construct (Figure 11 (c)).

The transgenic tobacco and tomato plants are then tested for their resistance against insects and micro-organisms. This is done by infesting control and transgenic plants with set numbers of pests and evaluating infestation of the plants after periods of 1, 2, 5, 7 and 14 days.

Example 8: Transformation of chicory with the germacrene A synthase gene(s) to obtain reduced bitterness

Based on the results described above, the sequences encoding germacrene A synthase are used to make transgenic plants showing reduced germacrene A synthase activity.

The down-regulation of genes in plants is achieved in different ways, e.g. through sense and anti-sense inactivation. In order to inhibit the activity of endogenous germacrene A synthases to obtain plant species with decreased production of germacrene A or products derived thereof, for example sesquiterpene lactones, plant species such as chicory and lettuce (and other sesquiterpene lactone producing species) are transformed by the techniques described above using plasmids containing genes coding for the germacrene A synthase(s) in either sense (to obtain co-suppression) or anti-sense orientation.

In order to test the impact of both genes on the production of sesquiterpenoids and the bitter taste of Chicory plants, constructs were made to inhibit either one or both germacrene A synthase genes together. Based on the expression pattern of the genes (Example 5) it was expected that for reduction of bitterness in the chicon, inhibition of the long gene alone could be sufficient.

The coding regions of the two genes encoding isoenzymes of germacrene A synthase of the present invention have a sequence similarity of 67%. Within the region encoding what is believed to be the "active domain" of the enzyme (stretching from about 40 amino acids before to about 140 amino acids behind the conserved DDXXD sequence) the sequence similarity between the two genes is 82.4%. Therefore, constructs are developed which comprise more than one DNA sequence whereby one DNA sequence comprises a DNA encoding an RNA with a sequence similarity to all or part of the sequence of SEQ ID NO 3, or the complementary strand thereof and the other encodes an RNA sequence with a sequence similarity to all or part of the sequence of SEQ ID NO 4, or the complementary strand thereof. The nucleic acid molecule used preferably comprises two sequences whereby the first sequence is identical to the complement of the second sequence, possibly separated by a spacer sequence, so as to form inverted repeats. The DNA sequences are placed under the control of adequate promoters, such as the 35S promoter, and terminator sequences and are introduced into chicory by Agrobacterium mediated transformation.

Examples of the DNA sequences comprise:

- the DNA of SEQ ID NO 3 and 4 (or parts thereof), behind a suitable promoter and upstream of a suitable terminator
- the DNA of SEQ ID NO 3 and 4 (or parts thereof), each behind a suitable promoter and upstream of a suitable terminator
- the DNA of SEQ ID NO 3 and 4 (or parts thereof), without a promoter
- two copies of SEQ ID NO 3 and 4 (or parts thereof) behind a promoter and upstream of a terminator
- a fragment of one of the two DNA's of SEQ ID NO 3 or SEQ ID NO 4 (or parts thereof) exhibiting a sequence similarity of preferably ≥80% to the other DNA of SEQ ID NO 3 or 4, behind a promoter and upstream of a terminator
- both DNA's of SEQ ID NO 3 and SEQ ID NO 4 (or parts thereof) and an additional upstream inverted copy of the 5' end (with or without the untranslated region) of one or of both, each behind a suitable promoter (or together behind one suitable promoter, such as a 35S promoter) and upstream of a suitable terminator (Hamilton et al., 1998, above; Waterhouse et al., 1998, above). Examples of such panhandle constructs are illustrated in Figure 11 (A, B, C).
- the cDNAs of SEQ ID NO 3 and SEQ ID NO 4, or a fragment thereof, displaying at least 70% homology to each other, whereby one is place in sense and one in anti-sense orientation, placed between a suitable promoter and terminator (Figure 11(D)).

The activity of the promoter, and hence the effectiveness of the co-suppression may be increased by including suitable enhancer elements behind the promoter, upstream of the coding sequence(s).

Chicory transformation: seeds were sterilized in a 2% sodiumhypochlorite solution during 20 minutes. After three rinses, the seeds were sown on MS20 in containers and grown at 27°C in light. Between 4 and 8 weeks after sowing the leaf-explants can be cut for transformation purposes. These explants (4x6mm) of which all sides are cut are ented with the backside onto the medium and pre-cultured for one day in MSN20 (Murashige & Skoog salts, Nitsch & Nitsch (1965) vitamins, 2.0 mg/l Glycine, 20 g/l sucrose, 8g/l agar, pH 5,8) medium containing 1mg/ml BA + 0.2 mg/ml NAA. The pre-cultured

leaf pieces were infected by submersing them for 20 minutes in a diluted bacterial suspension (OD 0.01 and 0.1). After removal of excess suspension the pieces were replaced on the pre-culture trays. After two days of co-cultivation, the leaf pieces were rinsed in MS20 medium for 30 minutes. They were then incubated on MSN20 containing 1 mg/ml BA and 0.1 mG/l NAA and 100 mg/l kanamycin (with 200 mg/l carbenicillin and 200 mg/l Claforan) at 27° in light. Selection for PPT was done on MSN medium containing 1mg/ml BA and 0.1 mg/l NAA and $2x10^{-5}$M DL-PPT (200 mg/l Carbenecillin and 200 mg/l Claforan). The leaf pieces were transferred to fresh selection medium every 14 days. About 5 weeks after cutting of the explants the selection medium was changed to MS 20 containing 0.1-1 mg/l BA and 100 mg/l kanamycin (200 mg/l Carbenecillin and 200 mg/l Claforan).

Chicory was transformed with constructs 1 and 4 (Figure 11), as well as the sense construct harboring the long DNA that was also used to transform tobacco and tomato (Example 7). After regeneration, leaf samples of 2-4 cm large regenerants were tested for germacrene A synthase activity as described in Example 7. For all three constructs there were a number of individuals with significantly reduced germacrene A synthase activity (and thus reduced amounts of bitter sesquiterpene lactones (Figure 12).

Claims

1. An isolated polynucleic acid comprising a nucleotide sequence encoding a protein or polypeptide having the biological activity of a germacrene A synthase.

2. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence encodes a protein or polypeptide with an amino acid sequence having at least 70% sequence similarity to the sequence of SEQ ID NO 7 or SEQ ID NO 8.

3. The isolated polynucleic acid of claim 2, wherein said nucleotide sequence encodes a protein or polypeptide with an amino acid sequence having at least 70% sequence similarity with the sequence of SEQ ID NO 7, between amino acid 271 and amino acid 455, or with the sequence of SEQ ID NO 8, between amino acid 291 and 477.

4. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence encodes the amino acid sequenc of SEQ ID NO 7.

5. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence encodes the amino acid sequence of SEQ ID NO 8.

6. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence has at least 70% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complement thereof.

7. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence has at least 95% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complement thereof.

8. The isolated polynucleic acid of claim 7, wherein said nucleotide sequence is the nucleotide sequence of SEQ ID NO 3, or the complement thereof.

9. The isolated polynucleic acid of claim 7, wherein said nucleotide sequence is the nucleotide sequence of SEQ ID NO 4, or the complement thereof.

10. A process for producing a plant with modified sesquiterpenoid synthase activity, said process comprising introducing into the genome of a plant cell a recombinant DNA which when expressed in a plant cell modifies the expression of a sesquiterpenoid synthase encoded by a polynucleic acid of any of claims 1 to 9 in said cell.

11. A process for producing a plant with reduced bitterness in some or all of its plant parts, said process comprising reducing the expression of an endogenous sesquiterpenoid synthase gene in said plant.

12. The process of claim 11, comprising
(a) introducing into the genome of a plant cell one or more recombinant DNAs, said recombinant DNAs comprising:
- a DNA encoding an RNA, protein or polypeptide, which when expressed in said plant cell inhibits or reduces the expression of an endogenous sesquiterpenoid synthase in said cell, and
- a plant-expressible promoter, whereby said DNA is in the same transcriptional unit and under the control of said plant-expressible promoter; and
(b) regenerating said plant from said plant cell or tissue

13. The process of claim 12, wherein said sesquiterpenoid synthase is a germacrene A synthase.

14. The process of claim 13, wherein said DNA encodes a sense or anti-sense RNA capable of inhibiting or reducing the expression of said endogenous germacrene A synthase.

15. The process of claim 14, wherein said DNA comprises a sequence having at least 70% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complementary strand thereof.

16. A process for producing a plant with increased pest resistance, said process comprising increasing or inducing the expression of a protein of polypeptide having germacrene A synthase in said plant or having the activity of an enzyme involved in the production of sesquiterpenoid lactones from germacrene A.

17. The process of claim 16, comprising

(a) introducing into the genome of a plant cell or tissue a recombinant DNA comprising:

- a DNA encoding a protein or polypeptide having germacrene A synthase activity, and

- a plant-expressible promoter; said DNA being in the same transcriptional unit and under the control of said plant-expressible promoter; and

(b) regenerating said plant from said plant cell or tissue

18. The process of claim 17, wherein said DNA encodes a protein or polypeptide having at least 70% sequence similarity to the amino acid sequence of SEQ ID NO 7 or with the sequence of SEQ ID NO 8.

19. The process of claim 17, wherein said DNA comprises a sequence having at least 70% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4.

20. The process of claim 17, wherein said DNA comprises the sequence of SEQ ID NO 3 or SEQ ID NO 4.

21. A recombinant polynucleic acid comprising
(a) a DNA encoding an RNA or protein, which when expressed in a cell of a plant either induces, increases or decreases the expression of germacrene A synthase in said cell, and
(b) a plant-expressible promoter; wherein said DNA is in the same transcriptional unit and under the control of said plant expressible promoter.

22. The recombinant polynucleic acid of claim 21, wherein said DNA encodes an antisense RNA, a ribozyme or a sense RNA, which when expressed in a cell of a plant decreases the expression of an endogenous germacrene A synthase in said cell.

23. The recombinant polynucleic acid of claim 21, wherein said DNA has at least 70% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complementary strand thereof.

24. The recombinant polynucleic acid of claim 23, wherein said polynucleic acid comprises the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or part thereof.

25. The recombinant polynucleic acid of claim 21, wherein said DNA comprises
- a first nucleotide sequence having at least 70% sequence similarity to the complementary sequence of SEQ ID NO 3 or SEQ ID NO 4, or part thereof, and
- a second nucleotide sequence having at least 70% sequence similarity to the sequence of SEQ ID NO 3 or SEQ ID NO 4 , or part thereof, and optionally,

- a spacer sequence between said first and said second sequence.

26. The recombinant polynucleic acid of claim 25, wherein said first nucleotide sequence is complementary to part of said second sequence.

27. The recombinant polynucleic acid of claim 21, wherein said DNA comprises
- a first nucleotide sequence having at least 70% sequence similarity to the sequence of SEQ ID NO 3 or SEQ ID NO 4, or part thereof, and
- a second nucleotide sequence having at least 70% sequence similarity to the sequence of SEQ ID NO 3 or SEQ ID NO 4, or part thereof.

28. The recombinant polynucleic acid of claim 21, wherein said DNA encodes a protein or polypeptide with germacrene A synthase activity.

29. The recombinant polynucleic acid of claim 28, wherein said DAN encodes a protein or polypeptide having at least 70% sequence similarity to the sequence of SEQ ID NO 7 or SEQ ID NO 8.

30. The recombinant polynucleic acid of any one of claims 21 to 29, wherein said plant-expressible promoter is the promoter of an endogenous germacrene A synthase gene.

31. The recombinant polynucleic acid of any one of claims 21 to 29, wherein said plant-expressible promoter is a constitutive promoter.

32. The recombinant polynucleic acid of any one of claims 21 to 29, wherein said plant-expressible promoter is an inducible or a tissue-specific promoter.

33. A cell of a plant, transformed with the recombinant polynucleic acid of any one of claims 21 to 32.

34. A plant consisting essentially of the plant cells of claim 33.

35. The plant of claim 34, which is selected from the group of the genera Carum, Chichorium, Daucus, Juniperus, Chamomilla, Lactuca, Pogstemon, and Vetivera.

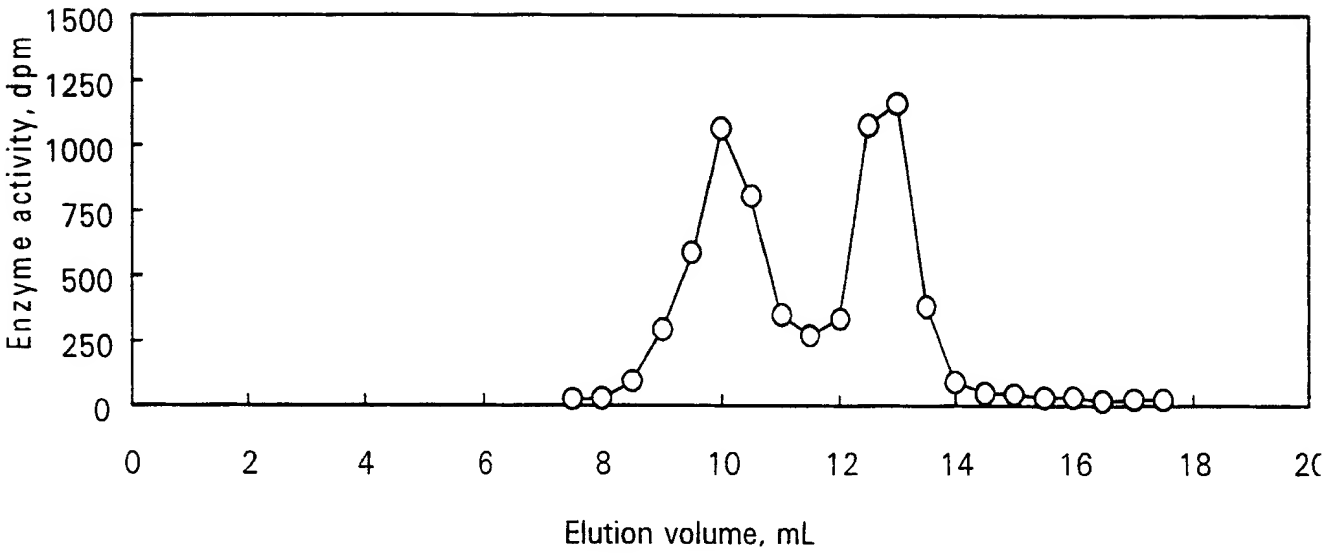
36. The seed of a plant of claim 35, comprising said recombinant DNA.

37. A probe which is part of a polynucleic acid sequence according to any of claims 1-9 and which hybridizes specifically with said polynucleic acid or the completement thereof.

38. A primer derived from a polynucleic acid sequence according to any of claims 1-9 and which specifically amplifies with said polynucleic acid or the completement thereof.

39. A process for producing a plant with reduced bitterness in some or all of its plant parts, said process comprising reducing the production of germacrene A opr a sesquiterpenoid lactone derived from germacrene A in said plant.

Figure 1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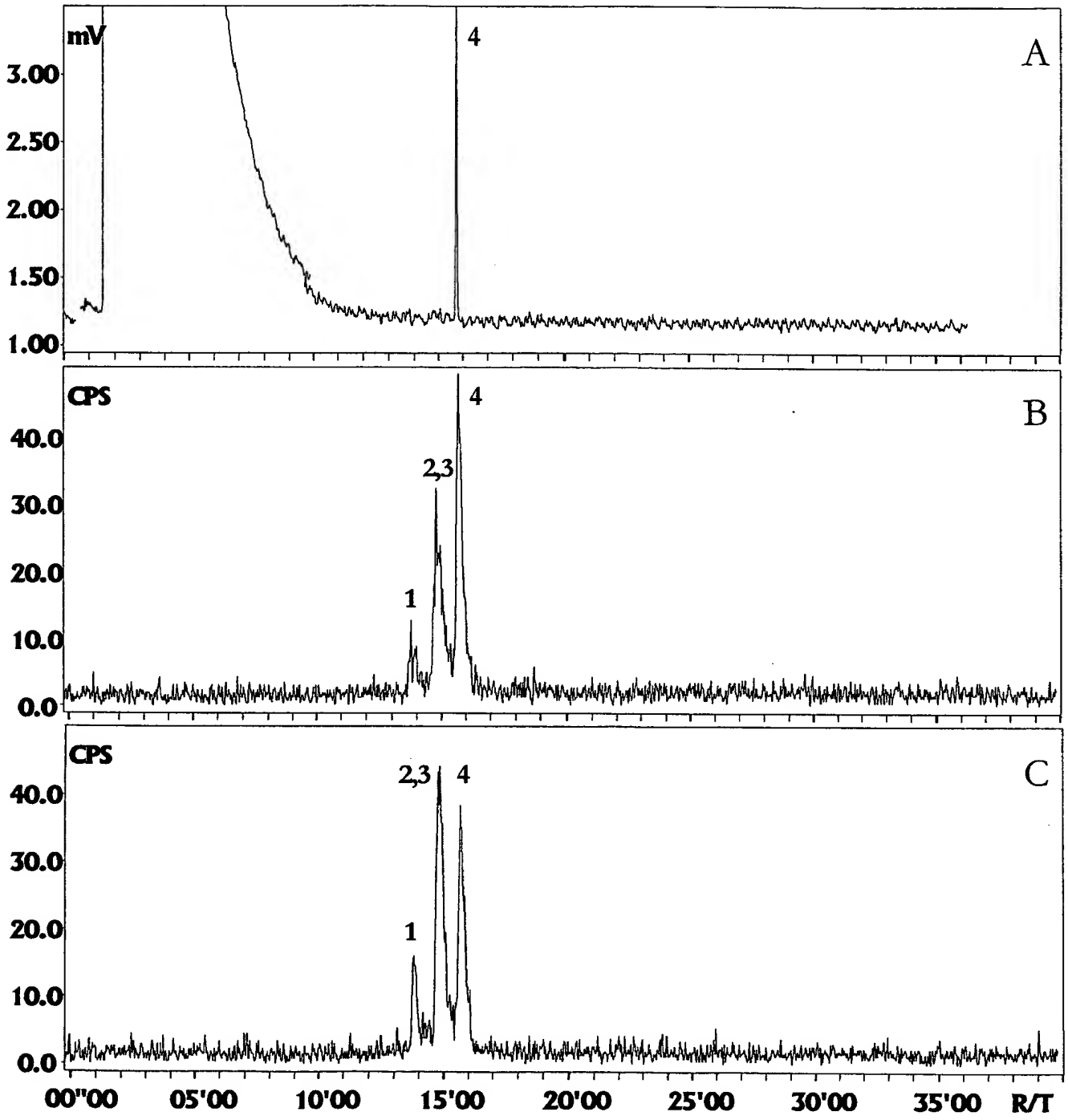
1250
1000
750
500
250
0
0
2
4
6
8
10
12
14
16
18
20
Elution volume, mL

Figure 3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2.00
1.50
1.00
4
A
CPS
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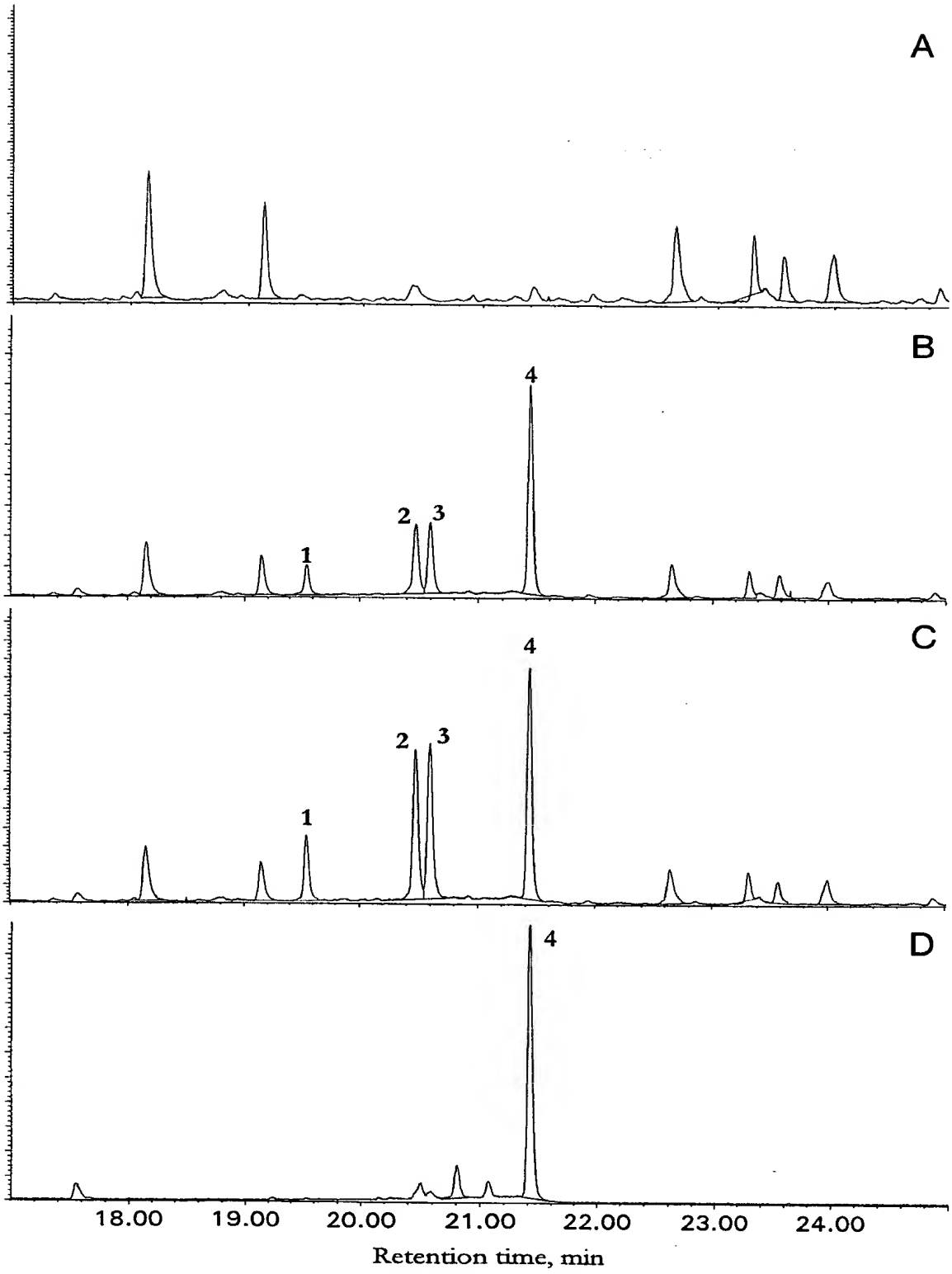
30.0
20.0
10.0
0.0
4
2,3
1
B
CPS
40.0
30.0
20.0
10.0
0.0
2,3
4
1
C
00''00
05'00
10'00
15'00
20'00
25'00
30'00
35'00
R/T

Figure 4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B
C
D
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2
3
4
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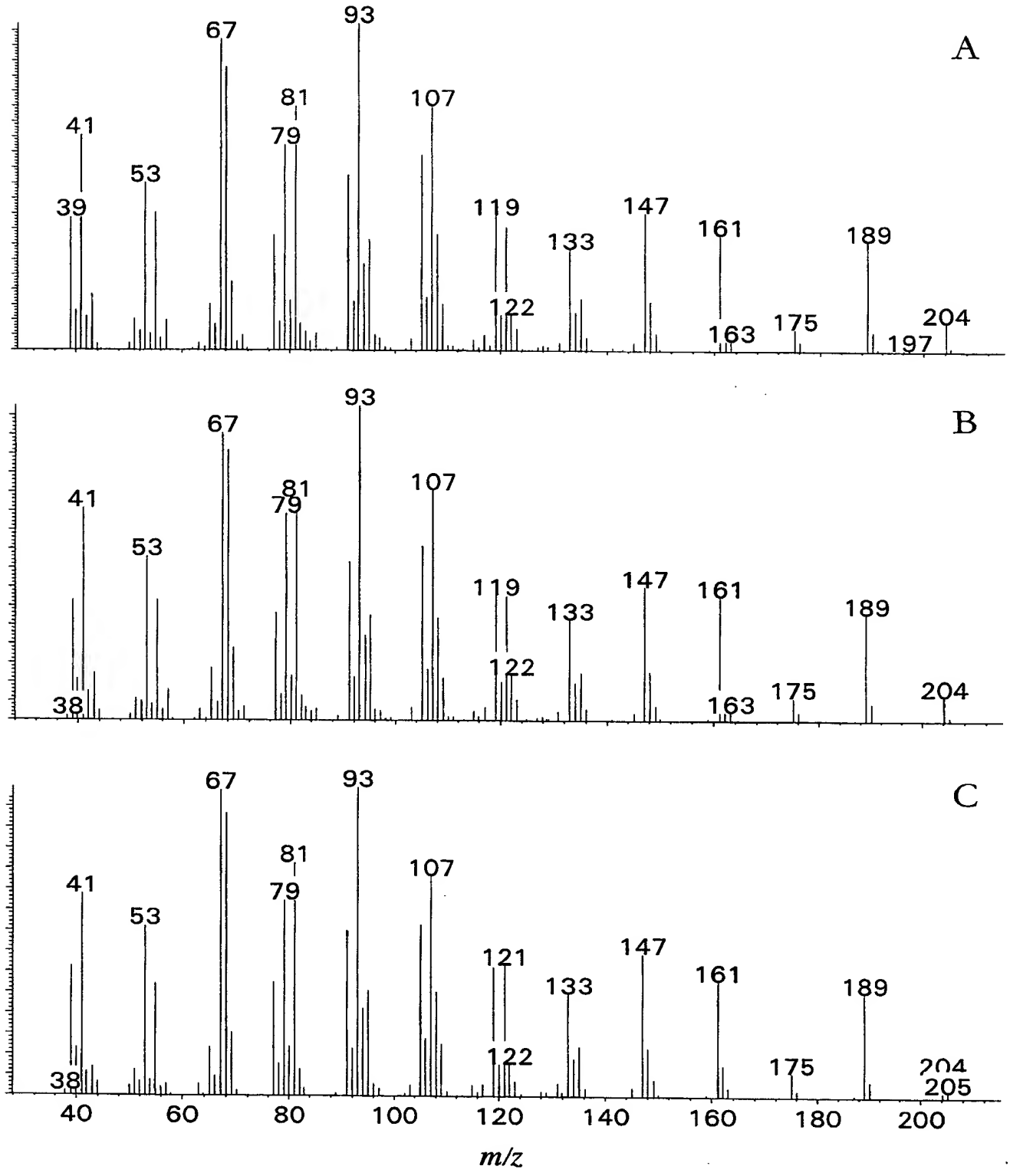
19.00
20.00
21.00
22.00
23.00
24.00
Retention time, min

Figure 5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81
107
41
79
53
39
119
147
161
133
189
122
175
204
163
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B
93
67
81
107
41
79
53
119
147
161
133
189
122
175
204
38
163
C
67
93
81
107
41
79
53
121
147
133
161
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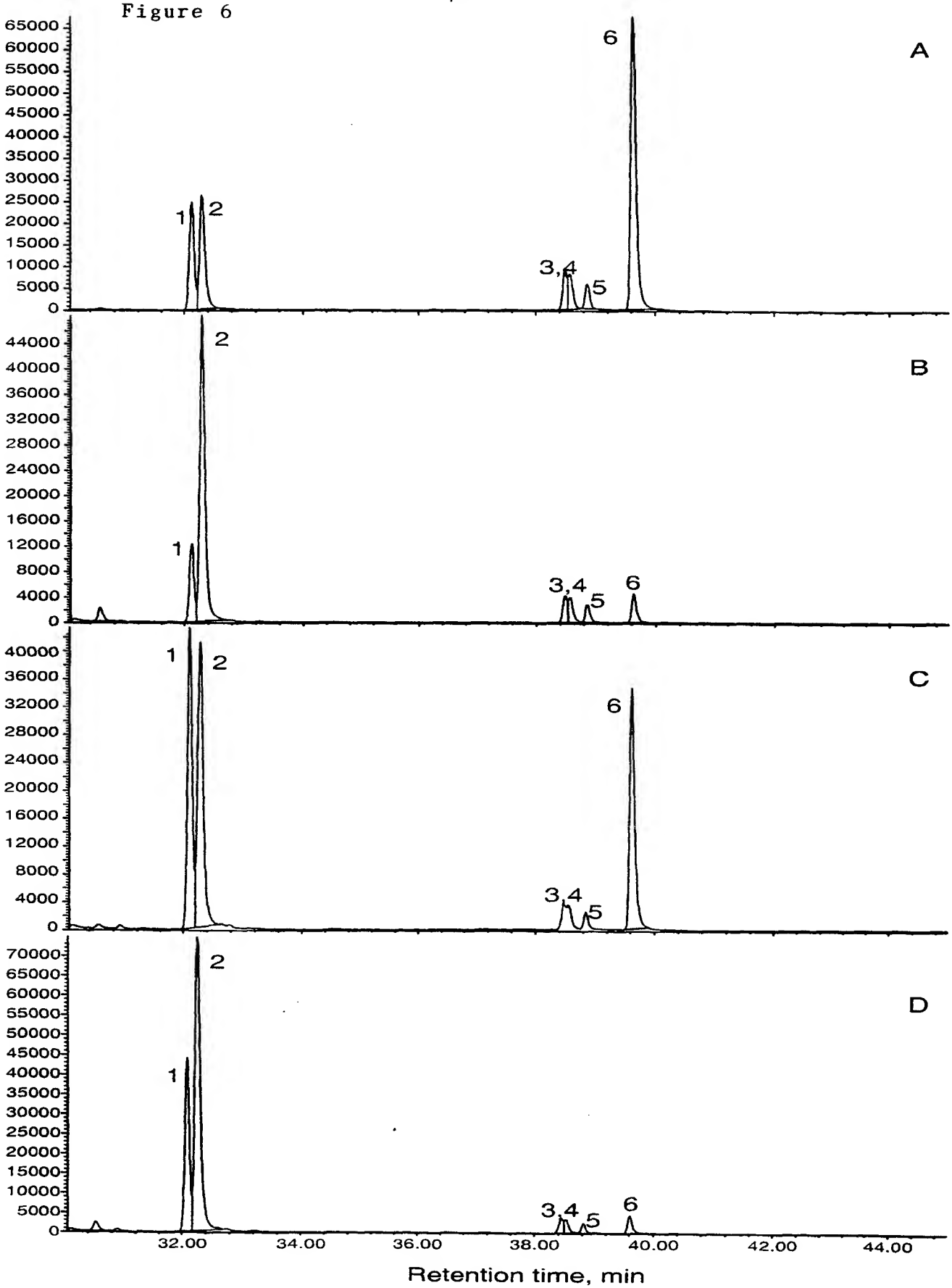
80
100
120
140
160
180
200
m/z

Figure 6


A
B
C
D
1
2
3,4
5
6
Retention time, min
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34.00
36.00
38.00
40.00
42.00
44.00

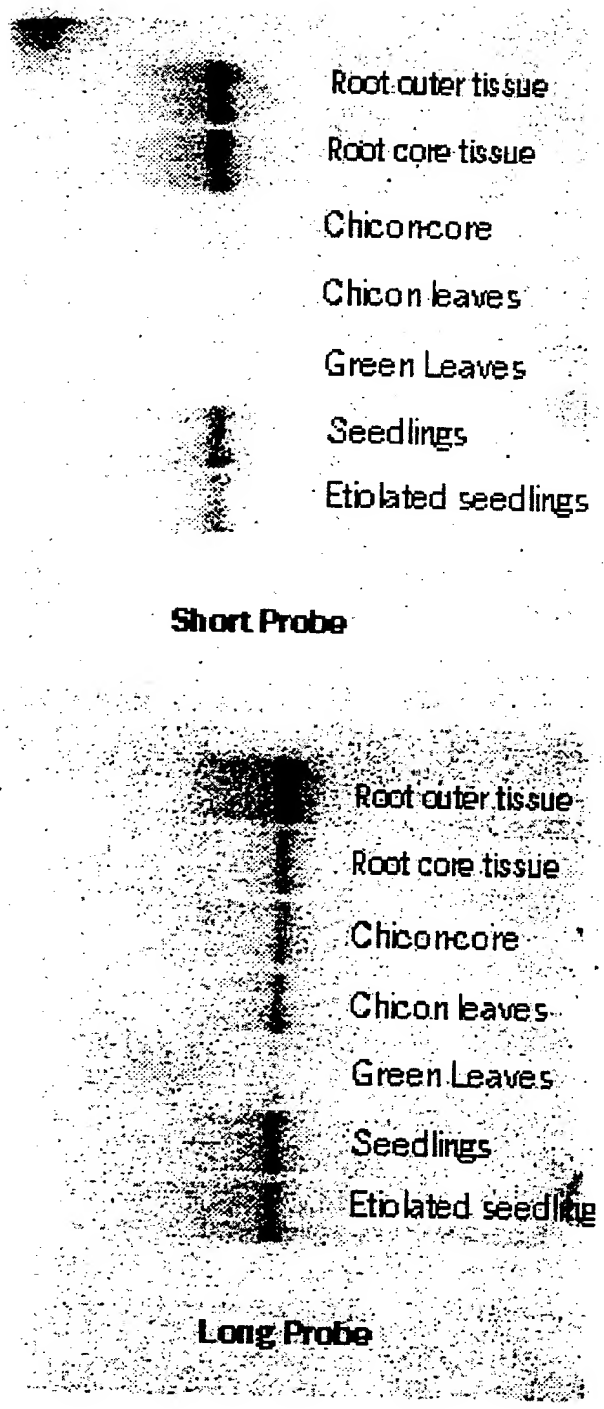

Root outer tissue
Root core tissue
Chicon core
Chicon leaves
Green Leaves
Seedlings
Etiolated seedlings
Short Probe
Root outer tissue
Root core tissue
Chicon core
Chicon leaves
Green Leaves
Seedlings
Etiolated seedlings
Long Probe

Figure 7 (a)

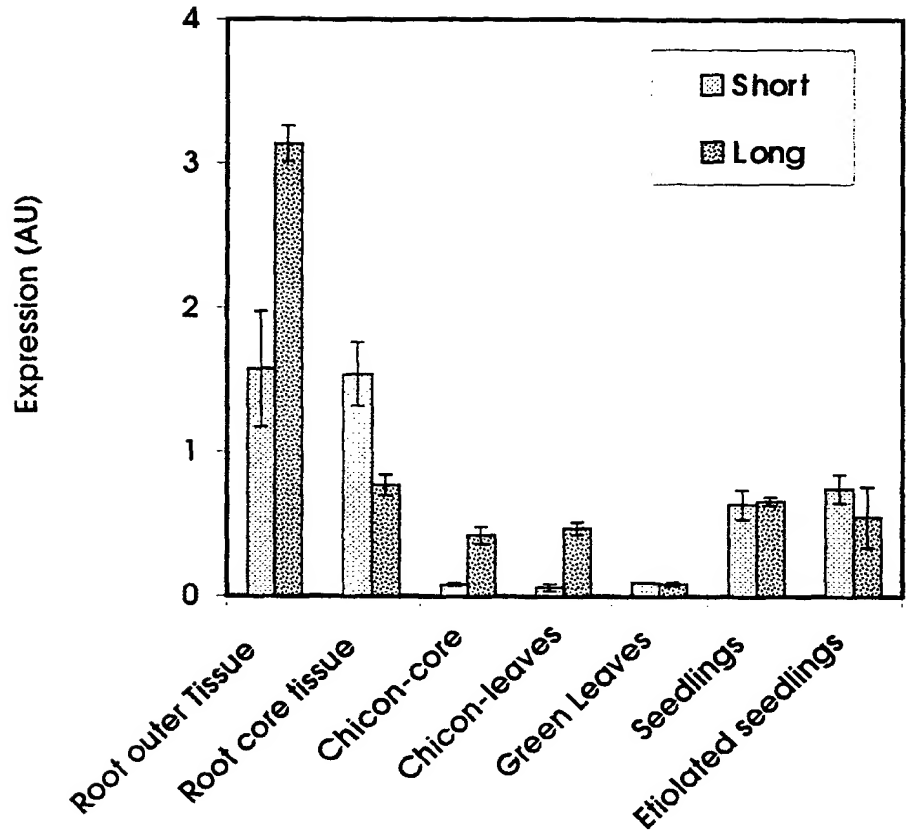

Expression (AU)
4
3
2
1
0
Short
Long
Root outer Tissue
Root core tissue
Chicon-core
Chicon-leaves
Green Leaves
Seedlings
Etiolated seedlings

Figure 7 (b)

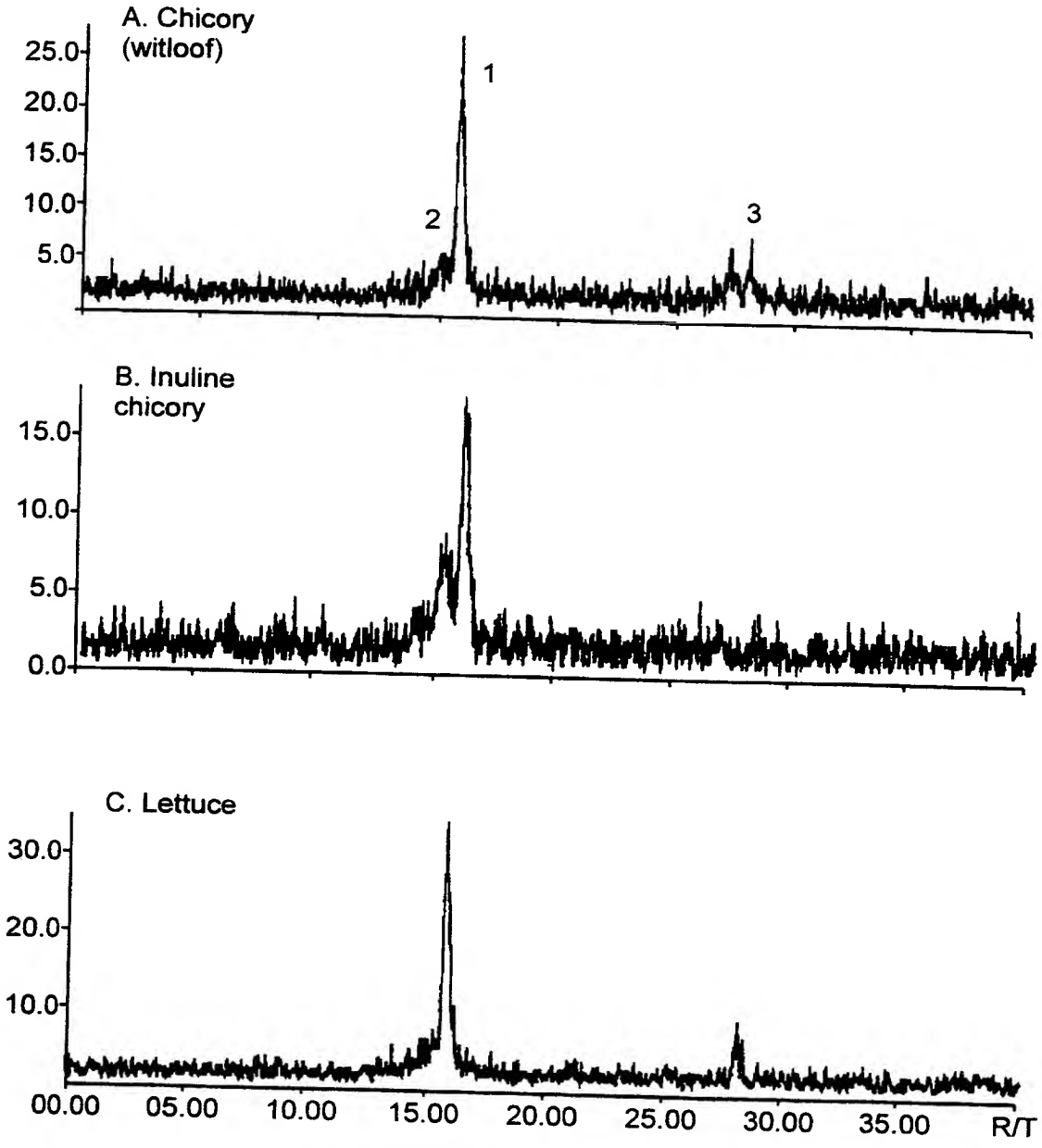


Figure 8 part 1

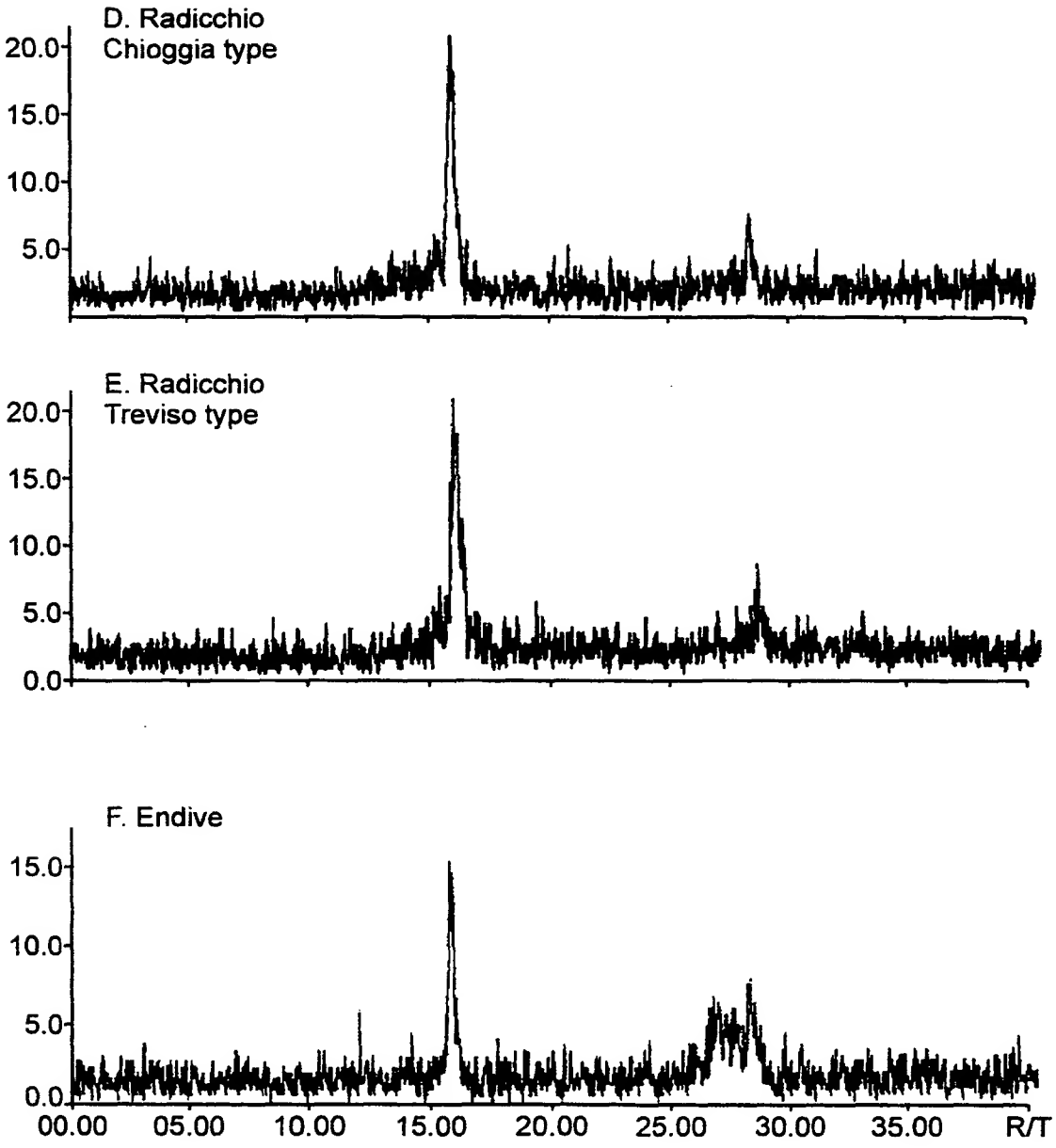
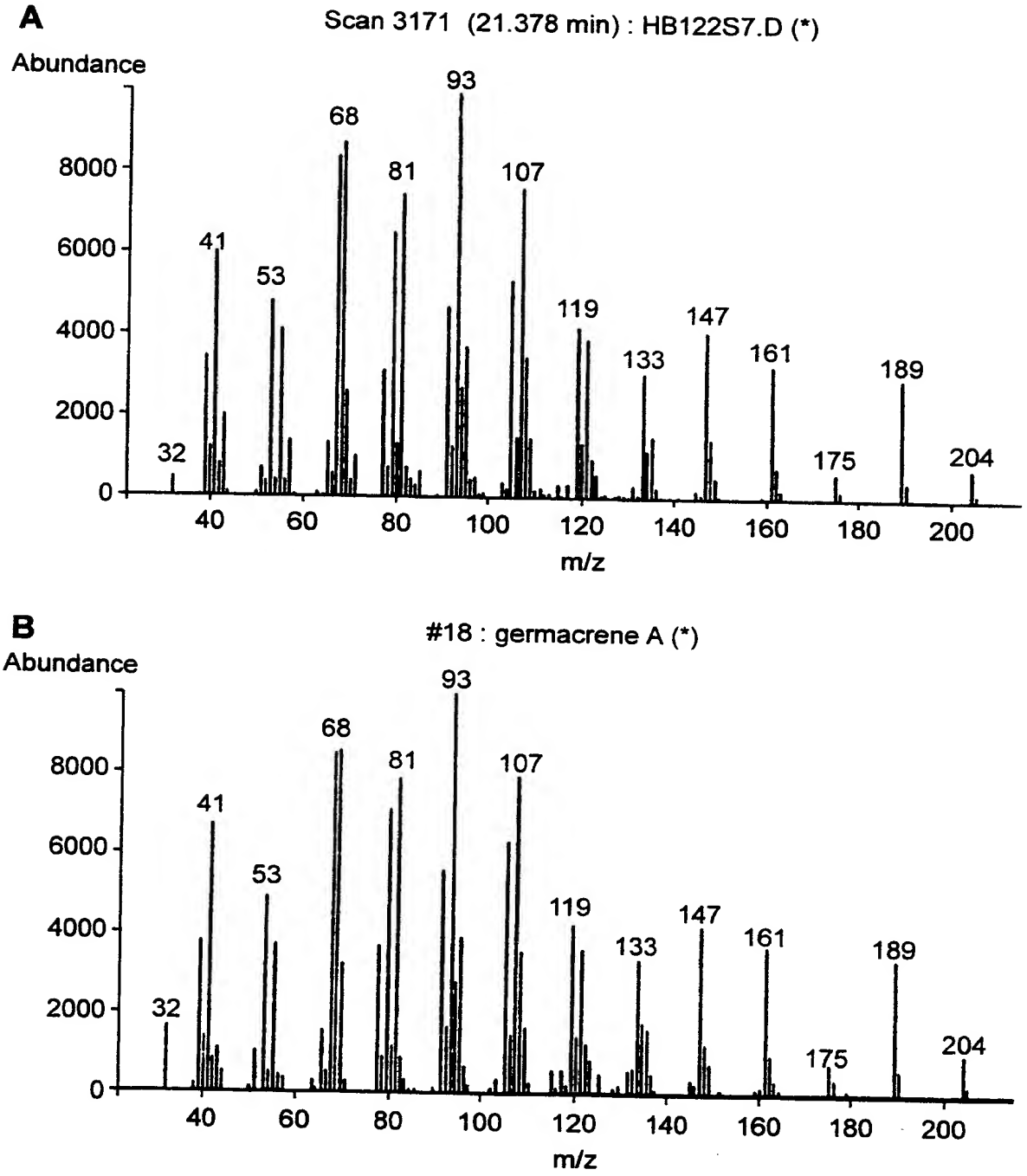


Figure 8 part 2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Scan 3171 (21.378 min) : HB122S7.D (*)
Abundance
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6000
4000
2000
0
32
41
53
68
81
93
107
119
133
147
161
175
189
204
40
60
80
100
120
140
160
180
200
m/z
B
#18 : germacrene A (*)
Abundance
8000
6000
4000
2000
0
32
41
53
68
81
93
107
119
133
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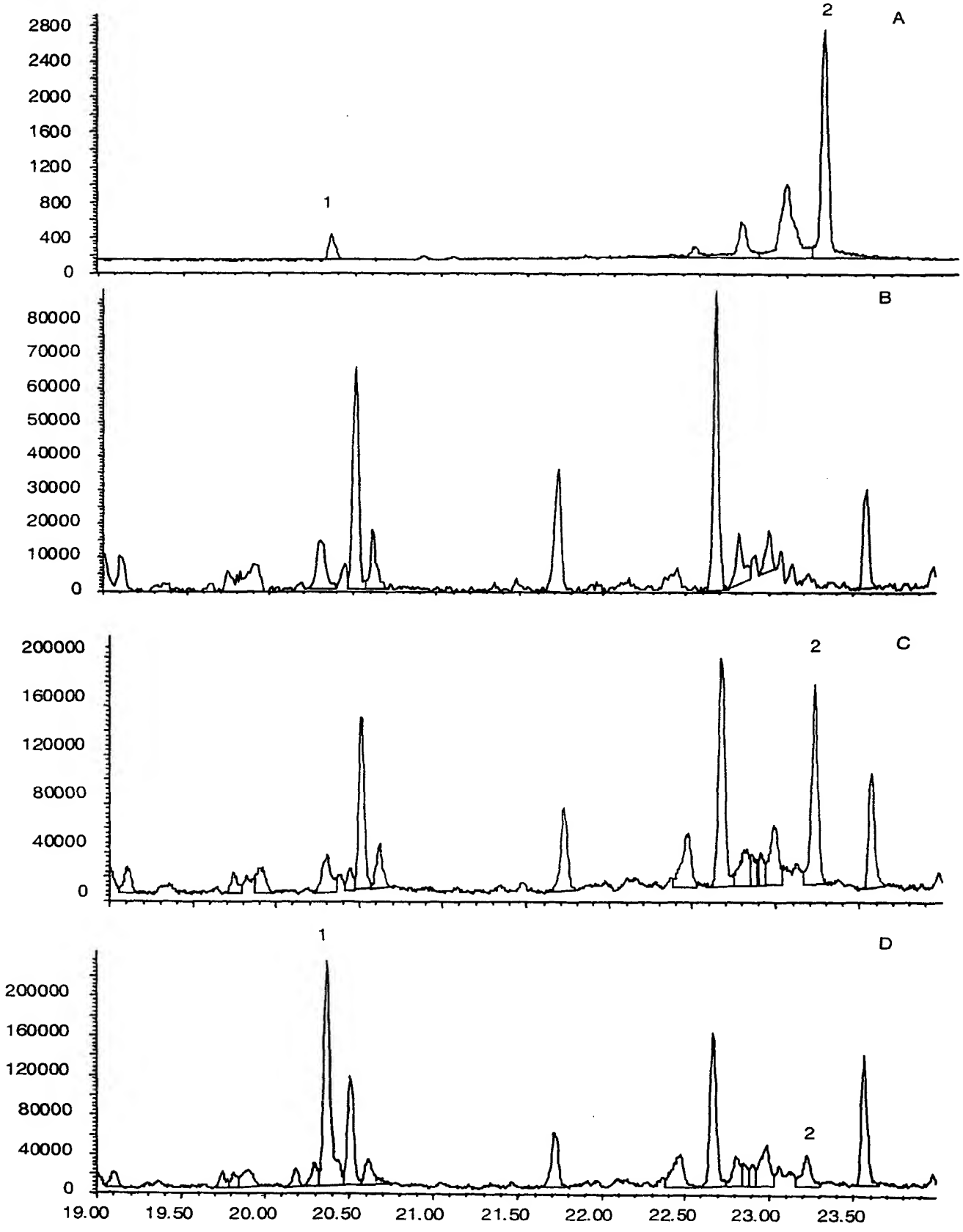
120
140
160
180
200
m/z

Figur 9

Figure 10a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B
C
D
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2
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2400
2000
1600
1200
800
400
0
80000
70000
60000
50000
40000
30000
20000
10000
200000
160000
120000
80000
40000
19.00
19.50
20.00
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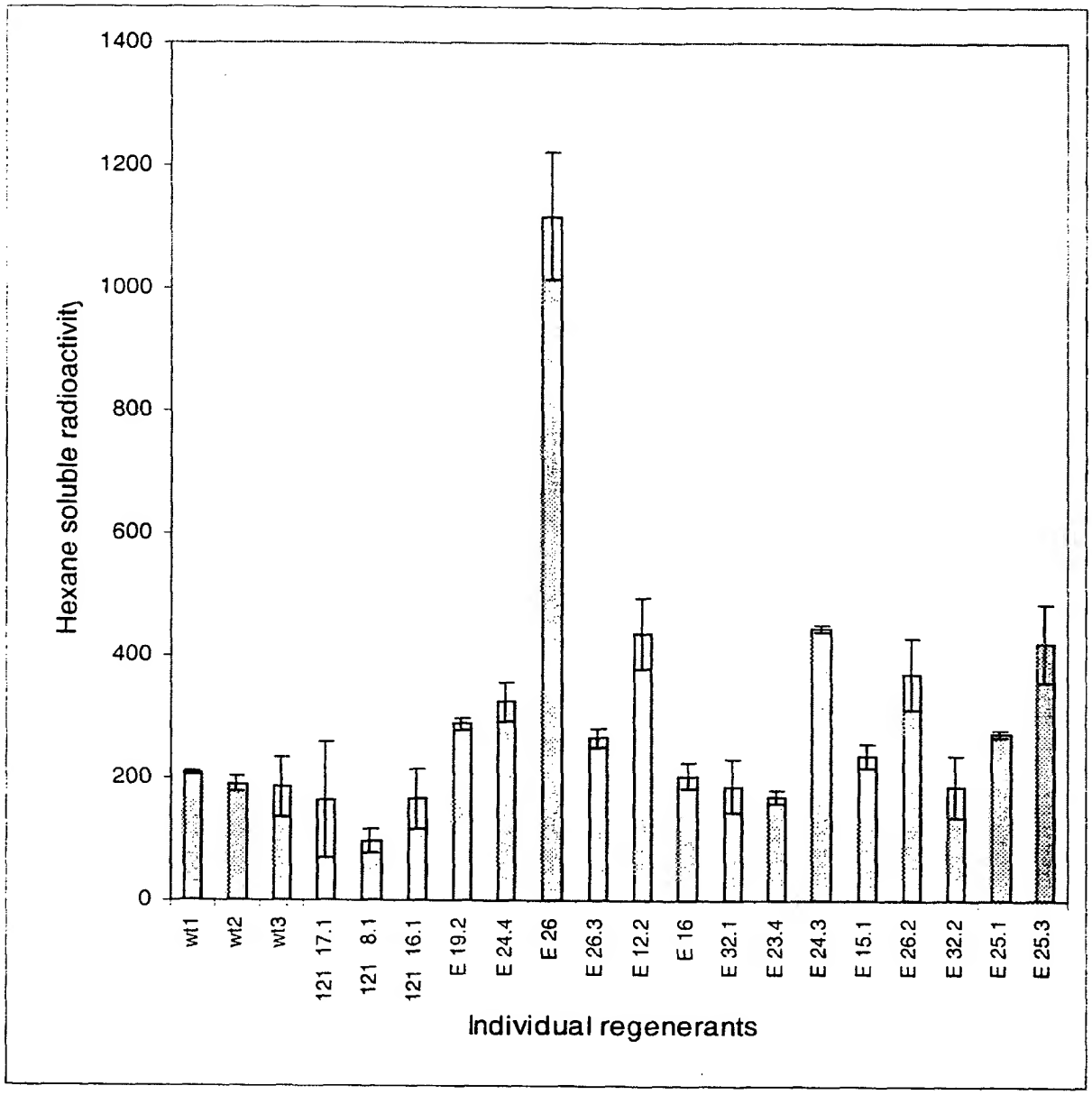
22.50
23.00
23.50
Retention time, min

Figure 10 (b)


Hexane soluble radioactivit)
1400
1200
1000
800
600
400
200
0
wt1
wt2
wt3
121 17.1
121 8.1
121 16.1
E 19.2
E 24.4
E 26
E 26.3
E 12.2
E 16
E 32.1
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E 26.2
E 32.2
E 25.1
E 25.3
Individual regenerants

Figure 10 (c)

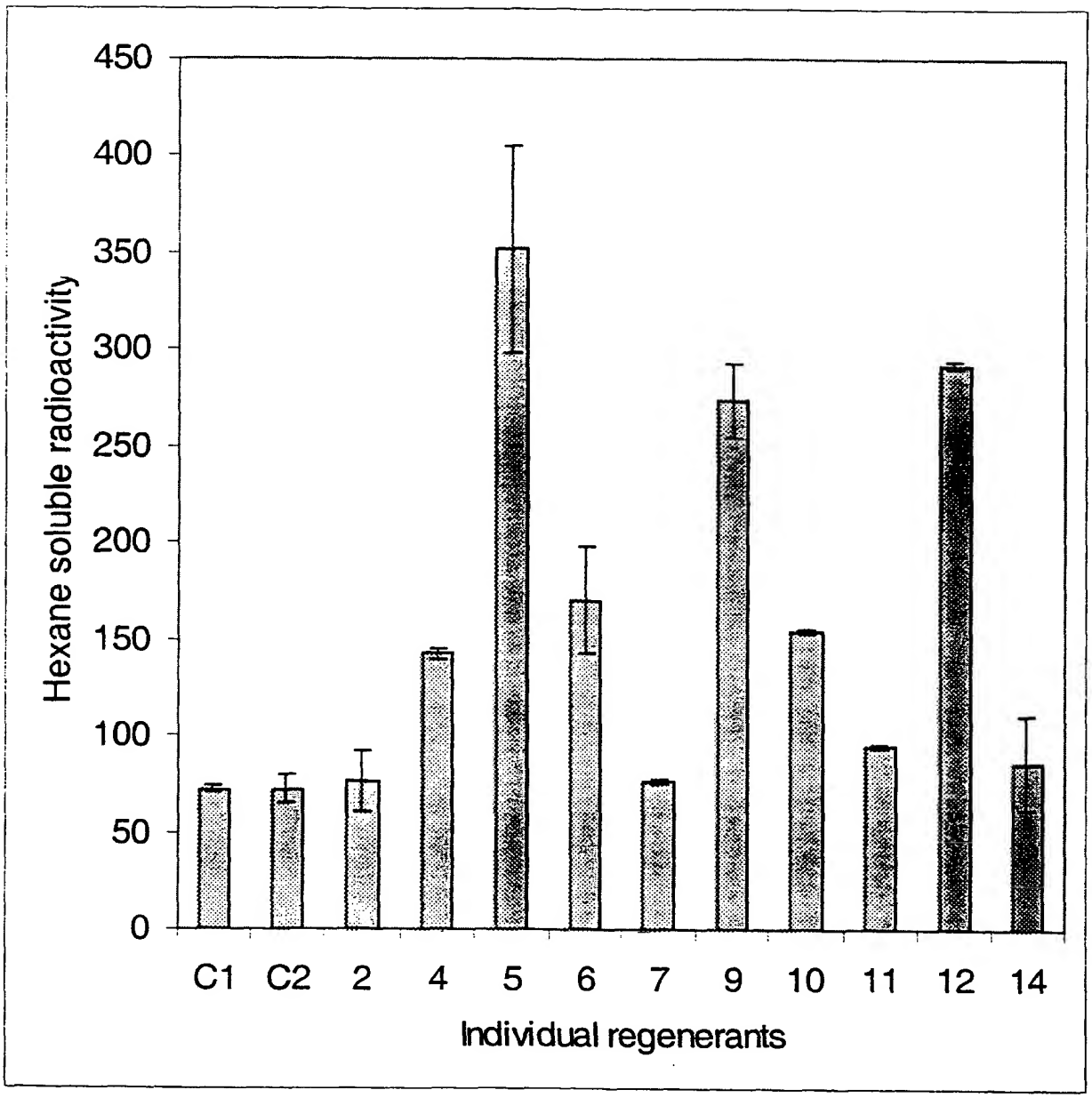
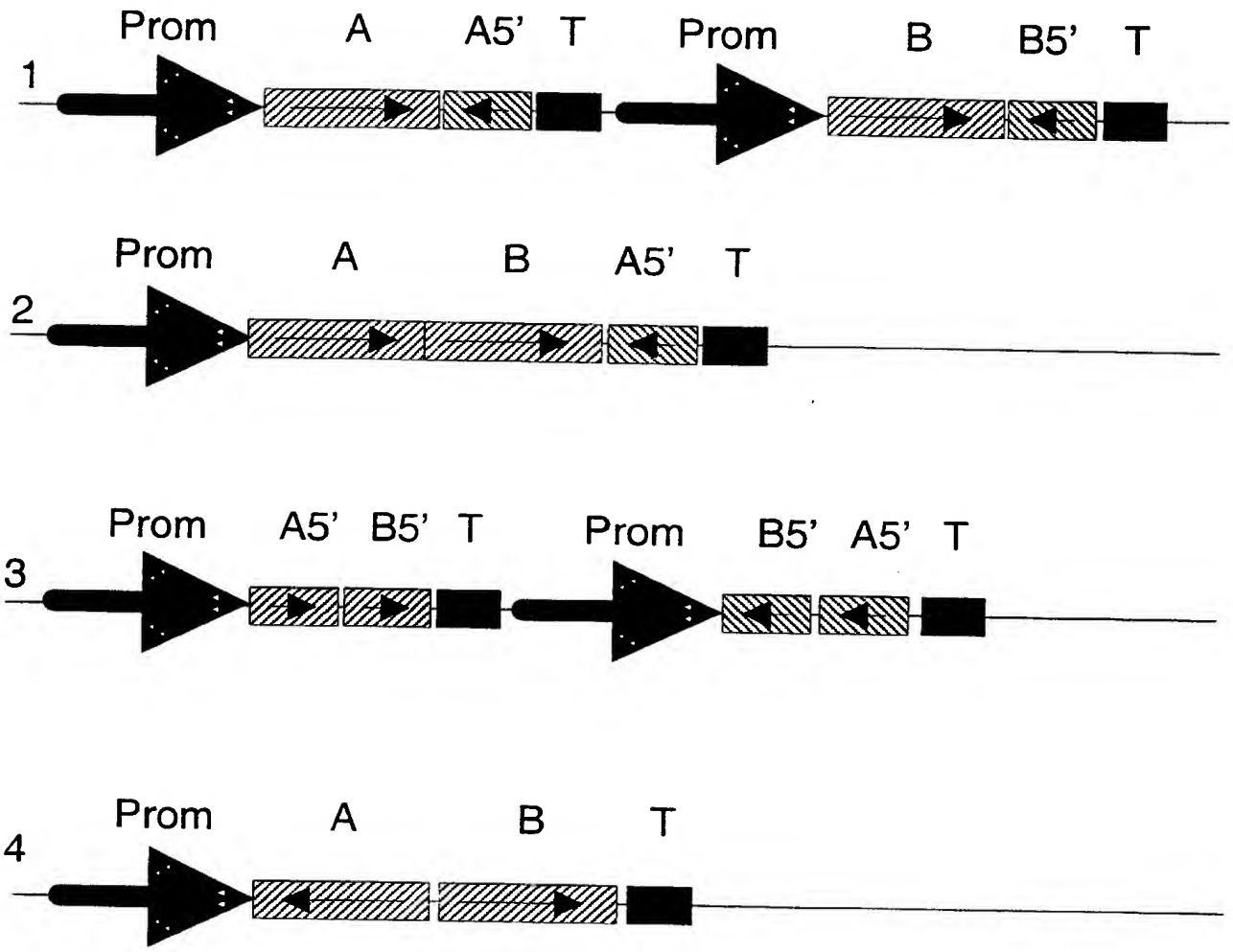

450
400
350
300
250
200
150
100
50
0
Hexane soluble radioactivity
C1
C2
2
4
5
6
7
9
10
11
12
14
Individual regenerants

Figure 11


1
Prom
A
A5'
T
Prom
B
B5'
T
2
Prom
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B
A5'
T
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Prom
A5'
B5'
T
Prom
B5'
A5'
T
4
Prom
A
B
T

Figure 12

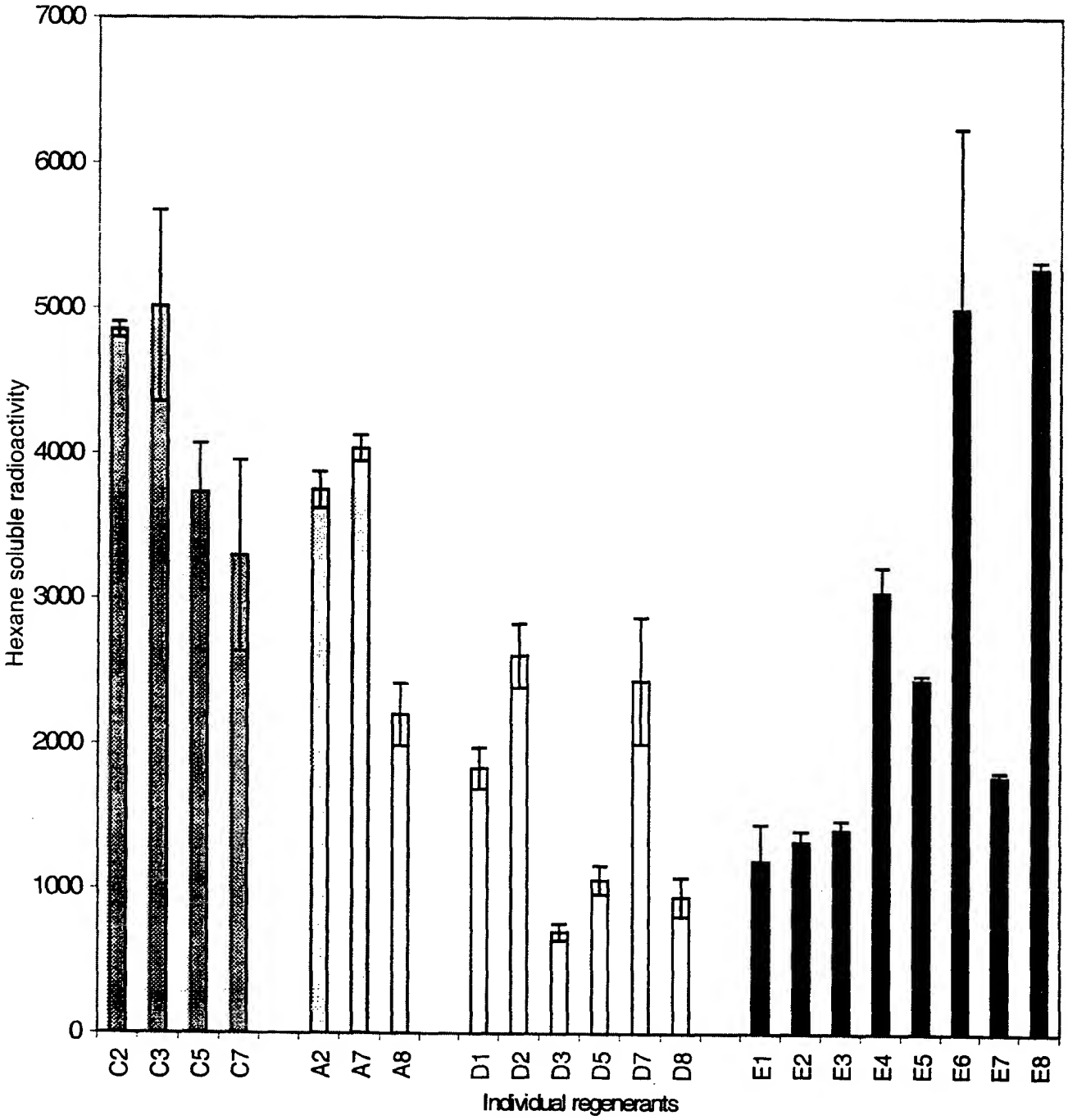
7000
6000
5000
4000
3000
2000
1000
0
Hexane soluble radioactivity
C2
C3
C5
C7
A2
A7
A8
D1
D2
D3
D5
D7
D8
E1
E2
E3
E4
E5
E6
E7
E8
Individual regenerants

Figure 13

```
A  -----ATCAAACTT-AGCTATATCCTT--------TCACCTT-----CAAGA-----AAT   36
B  GTTTCATTAAAGATCATCCA-ATCCGAAAAATGGCTCTCGTTAGAAACAACAGCAGCAAT   59
        ** ***  * * * * ****          ** * **     *** *     ***

A  GGCAG-----CAGTT----------GAAGCCA-ACGGTACCTTCCAAGC--AAACA----   74
B  GGTCGGGAGCCAGTTCTCAGCCCCAGAAGTCTCACAAGTCCT-CGAGGCTTAACCAGCCC  118
   **  *     *****          **** *  **    *** * * **  ** **

A  ---------------CCAAA--ACCACAGAGCCGGTGCGTCCTTTAGCCAACTTCCCTCC  117
B  CCGACCGTTGTCTGTCCAACCGACTCCAGAGCCGGTTCGACCTTTGGCCAACTTCCCACC  178
                  ****   **  ********** ** ***** *********** **

A  TTCTGTATGGGGTGATCGATTCTTGTCATTCTCTCTTGACACTACGGAATTGGAAGGATA  177
B  TTCGATCTGGGCTGATCGCTTCATCTCATTCTCTCTTGATAACTCTCAATTGGAAGCTTA  238
   ***  * **** ****** *** * ************** *   *  *********  **

A  TGCAAAAGCTATGGAGGAGCCAAAAGAAGAAGTGAGAAAACTGATCGTAGATCCAACAAT  237
B  TGCAAATGCACTTGAAGAACCAAAAGAAGCAGTGAAGAGTTTAATAACCGACACTACCAT  298
   ****** **  * ** ** ********** *****  *   * **    **  * ** **

A  GGATTCAAATAAGAAACTAAGTTTGATTTATTCTGTACACCGTCTTGGTTTGACATATCT  297
B  TGATGCAAACACAAAACTGAAATTGATTTATTCAGTGCACCGTCTTGGTTTGTCGTATCT  358
    *** **** *  ***** *  *********** ** *************** * *****

A  GT-TCTTGCAAGAGATTGAAGCGCAGCTTGACAAACTATTTAAAGAGTTTAACTTGCAAG  356
B  TTATCCAG-ATGAGATTGATGCCGAACTCAACAAACTCTTCGAGAAGATTGACTTACAGT  417
    * **  * * ******** **  * **  ******* **  *  ** ** **** **

A  ATTATGATGAGTTTGATCTATACACAACTTCTATTAACTTTCAAGTTTTCAGACACCTTG  416
B  ATTACGAACAAGTTGATTTGTACACTATTGCAGTACAATTTCAAGTTTTCAGACACCATG  477
   **** **  *  ***** * ***** * * *  *  * ******************* **
```

Figure 13 (continued)

```
A   GTCACAAACTGCCATGTGATGTGTTTAACAAATTCAAGGACAGTAGCTCGGGTACATTCA   476
B   GTTATAAAATTTCTTCTGATGTGTTTAAAAAGTTCAAGGACAGTACTACGGGTACATTCA   537
    ** * *** *  * * ************ ** *************   ************

A   AGGAATCCATTACCAACGATGTGAAGGGTATGTTAGGCTTATATGAATCTGCACAATTGA   536
B   CGGATGATGTAACAAAAGACGTGAAGGGTATGCTGAGTTTATACGAATCAGCACACTTAA   597
     ***     * ** ** ** ************ *  * ***** ***** ***** ** *

A   GATTAAGAGGAGAACCCATTCTCGATGAAGCTTCCGCATTCACTGAAACTCAACTCAAGA   596
B   GGCTACACGGTGAAGATATCTTAGATGAAGCTTTAGCATTCACCGAAGCTCACCTTAAAA   657
    *  **   ** ***   **  * **********  ******** *** **** ** ** *

A   GTGTAGTAAACACTCTCGAAGGCAATCTTGCAAAACAGGTGATGCAATCATTGAGGAGAC   656
B   AAATCCTAACCACACTTGAAGGAGATCTTGCACGCCAAGTGAACCAAGTCTTAAAAAGAC   717
       *  *** *** ** *****  ********   ** ****  ***   ** *  ****

A   CATTCCATCAGGGGATGCCAATGGTTGAGGCAAGGATGTATTTCTCCAACTATGATGAAG   716
B   CCTTCCACACTGGAATGCCAATGGTAGAGGCACGGCTATATTTTATCACACACGAAGAAG   777
    * *****    ** *********** ****** ** * *****   **   * ** ****

A   AATGTTCCACACACGAGTCATTACCTAAGCTTGCAAAGCTTCATTTCAACTATTTGCAGC   776
B   ATTTTTCAAGCCATGAGTCGGTTGTAAAGCTAGCTAAAGTCCACTTCAACTATTTGCAAC   837
    * * *** *  ** *****  *    ***** ** **  * ** ************** *

A   TACAACAAAAGGAAGAACTTCGCATTGTCTCAAAGTGGTGGAAGGATATGAGGTTCCAGG   836
B   TACAACAAAAAGAAGAACTACGACTCGTGTCACAGTGGTGGAAAGATATGCAATTCCAAC   897
    ********** ******** **  * ** *** ********** ******   *****

A   AAACTACTCCTTACATAAGGGATAGAGTACCAGAGATTTACTTATGGATATTGGGATTGT   896
B   AATCCGTCCCTTACATAAGAGATAGAGTACCGGAGATATACCTATGGATTTTGGGGTTAT   957
    ** *    *********** *********** ***** *** ******* ***** ** *
```

Figure 13 (continued)

```
A  ACTTTGAGCCTCGTTACTCCTTGGCACGAATCATCGCCACAAAAATTACATTGTTCCTCG  956
B  ATTTCGAGCCGTATTACTCTCGGGCACGTATCATAGCCACTAAAATCACGTTGTTCTTGG 1017
   * ** *****   ******   ****** ***** ***** ***** ** ****** * *

A  TGGTTCTAGATGACACATATGATGCATACGCTACCATTGAAGAGATTCGACTTCTGACTG 1016
B  TGGTTTTGGACGATACATATGACGCGTATGCTACAATTGACGAGATCCGATCGATCACAG 1077
   ***** * ** ** ******** ** ** ***** ***** ***** ***    * ** *

A  ATGCCATAAACAGGTGGGACATCAGTGCTATGGAGCAAATTCCGGAATATATTCGACCAT 1076
B  ATGCGATTAATAGGTGGGAAATTAGCGCGATCGACCAACTTCCTGAATATATCAAACCGT 1137
   **** ** ** ******** ** ** ** ** ** *** **** ********   *** *

A  TCTACAAAATTCTCCTAGATGAGTATGCTGAACTTGAGAAGCAACTAGCTAAAGAAGGAA 1136
B  TCTACAGAATTCTCCTCAACGAATATGATGACCTCGAGAAAGAATACTCAAAGGACGGAA 1197
   ****** *********  * ** **** *** ** *****   **    * ** ** ****

A  GAGCAAAAAGTGTTATTGCTTCAAAAGAAGCGTTCCAGGACATTGCAAGAGGATACCTTG 1196
B  GAGCGTTCAGTGTCCACGCTTCAAAACAAGCGTTTCAAGAAATCGCGAGAGGGTATCTTG 1257
   ****    *****    ********* ******* ** ** ** ** ***** ** ****

A  AAGAGGCCGAGTGGACAAACAGTGGATACGTGGCATCATTTCCAGAGTATATGAAGAACG 1256
B  AAGAGGCGGAGTGGTTACACAACGGTTATGTGGCAACATTTCCCGAGTATATGAAGAATG 1317
   ******* ******  * ***  ** ** ****** ******* ************** *

A  GTTTAATTACTTCTGCTTACAATGTTATTTCAAAATCTGCTTTAGTGGGTATGGGCGAGA 1316
B  GTTTGATTACTTCGGCTTATAATGTCATTTCAAAATCCGCATTGGTGGGAATGGGTGCGA 1377
   **** ******** ***** ***** *********** ** ** ***** ***** * **

A  TGGTTGGTGAAGATGCCTTGGCTTGGTATGAAAGTCATCCAAAGACATTGCAAGCTTCAG 1376
B  TTGCAGATGAAGAGGCTCTTGCTTGGTATGAAACACATCCGAAAATTTTGAAAGCTTCAG 1437
   * *  * ****** **  * *************  ***** ** *  *** *********
```

Figure 13 (continued)

```
A   AGTTAATTTCAAGACTCCAAGATGATGTGATGACTTACCAGTTTGAGCGAGAAAGGGGAC 1436
B   AGTTGATTTCAAGGCTCCAAGACGATGTTATGACTTTCCAGTTTGAGAGAAAACGAGGAC 1497
    **** ******** ******** ***** ******* ********** ** ** * ****

A   AATCAGCCACCGGCGTTGATTCTTATATCAAGACCTATGGCGTAACAGAAAAGGAAGCGA 1496
B   AATCAGCAACTGGTGTGGATGCTTATATCAAGGAATACAATGTATCCGAAGAAGTAGCGA 1557
    ******* ** ** ** *** ***********   **    *** * *** * * *****

A   TTGACGAGCTAAACAAAATGATTGAAAATGCATGGAAGGATATAAATGAGGGCTGCCTTA 1556
B   TCAAAGAGCTCATGAAGATGATTGAAAACGCATGGAAAGATATAAATGAGGGATGCTTGA 1617
    *  * ***** *  ** ********** ******** ************** *** * *

A   AGCCAAGAGAAGTCTCAATGGATTTGCTTGCCCCAATTCTTAATCTTGCACGAATGATAG 1616
B   AGCCCACTGAGGTCTCGGTGGCTCTACTAACTCCTATTTTGAATCTCGCGAGAATGATAG 1677
    **** *  ** *****  *** * * **  * ** *** * ***** **  *********

A   ATGTGGTATACAGGTACGACGATGGGTTCACTTTTCCGGGAAAGACCATGAAAGAGTATA 1676
B   ATGTCGTATACAAATTCGATGATGGATTCACCTTTCCCGGGAAAACCCTAAAAGACTATA 1737
    **** *******  * *** ***** ***** ***** ** ** *** * ***** ****

A   TTACTCTGTTGTTTGTTGGTTCTTCACCCA-------------------TGTAATA---- 1713
B   TTACCCTTTTGTTCGTTAGTCCTCCACCGAGTCTCGAAAACTGATAGGTTGTAATATAAG 1797
    **** ** ***** *** ** ** **** *                    *******

A   ----ATA----------ATTTT--TATTTCAT-------ATGTTT--------------- 1735
B   CATCATATTTTCTCGGAATTTTGCTTTTTCATGTTGCGTATGTTTGCGACTTTCGCTCAA 1857
        ***          *****  * ******       ******

A   ------------------------------------------------------------
B   TTTGGTGAGATTCTTTTTGACATTCTATTGTATAATCTTCTTTTGTTCAAATAAAAAGTT 1917
```

Figure 13 (continued)

```
A   --------------AAAAAAAAAAAAAAAAAAAA-- 1755
B   TCTTCTTATGGACTAAAAAAAAAAAAAAAAAAAAAA 1953
                  ********************
```

Figure 14

```
A    MALVRNNSSNGREPVLSPRSLTSPRGLTSPRPLSVQPTPEPVRPLANFPPSIWADRFISF  60
B    MAAVEAN------------------GTFQAN----TKTTEPVRPLANFPPSVWGDRFLSF  38
     ** *. *                   *  ...      *.************:*.***:**

A    SLDNSQLEAYANALEEPKEAVKSLITDTTIDANTKLKLIYSVHRLGLSYLYPDEIDAELN 120
B    SLDTTELEGYAKAMEEPKEEVRKLIVDPTMDSNKKLSLIYSVHRLGLTYLFLQEIEAQLD  98
     ***.::**.**:*:***** *:.**.*.*:*:*.**.**********:**: :**:*:*:

A    KLFEKIDLQYYEQVDLYTIAVQFQVFRHHGYKISSDVFKKFKDSTTGTFTDDVTKDVKGM 180
B    KLFKEFNLQDYDEFDLYTTSINFQVFRHLGHKLPCDVFNKFKDSSSGTFKESITNDVKGM 158
     ***:::**  *:: .**** :::****** *:*:..***:*****::***.:.:*:*****

A    LSLYESAHLRLHGEDILDEALAFTEAHLKKILTTLEGDLARQVNQVLKRPFHTGMPMVEA 240
B    LGLYESAQLRLRGEPILDEASAFTETQLKSVVNTLEGNLAKQVMQSLRRPFHQGMPMVEA 218
     *.*****:***:** ***** ****::**.::.****:**:** * *:**** *******

A    RLYFITHEEDFSSHESVVKLAKVHFNYLQLQQKEELRLVSQWWKDMQFQQSVPYIRDRVP 300
B    RMYFSNYDEECSTHESLPKLAKLHFNYLQLQQKEELRIVSKWWKDMRFQETTPYIRDRVP 278
     *:** .::*: *:***: ****:**************:**:*****:**::.********

A    EIYLWILGLYFEPYYSRARIIATKITLFLVVLDDTYDAYATIDEIRSITDAINRWEISAI 360
B    EIYLWILGLYFEPRYSLARIIATKITLFLVVLDDTYDAYATIEEIRLLTDAINRWDISAM 338
     ************* ** *************************:*** :*******:***:

A    DQLPEYIKPFYRILLNEYDDLEKEYSKDGRAFSVHASKQAFQEIARGYLEEAEWLHNGYV 420
B    EQIPEYIRPFYKILLDEYAELEKQLAKEGRAKSVIASKEAFQDIARGYLEEAEWTNSGYV 398
     :*:****:***:***:** :***: :*:*** ** ***:***:********** :.***
```

Figure 14 (continued)

```
A    ATFPEYMKNGLITSAYNVISKSALVGMGAIADEEALAWYETHPKILKASELISRLQDDVM 480
B    ASFPEYMKNGLITSAYNVISKSALVGMGEMVGEDALAWYESHPKTLQASELISRLQDDVM 458
     *:************************** :..*:******:*** *:*************

A    TFQFERKRGQSATGVDAYIKEYNVSEEVAIKELMKMIENAWKDINEGCLKPTEVSVALLT 540
B    TYQFERERGQSATGVDSYIKTYGVTEKEAIDELNKMIENAWKDINEGCLKPREVSMDLLA 518
     *:****:*********:*** *.*:*: **.** ***************** ***: **:

A    PILNLARMIDVVYKFDDGFTFPGKTLKDYITLLFVSPPPSLEN    583
B    PILNLARMIDVVYRYDDGFTFPGKTMKEYITLLFVGSSPM---    558
     *************::**********:*:*******...*
```

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/EP 00/02130

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/52 C12N9/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLBY S M ET AL.: "Germacrene C synthase from Lycopersicum esculentum cv. VFNT cherry tomato: cDNA isolation, characterization, and bacterial expression of the multiple product sesquiterpene cyclase" PROCEEDING OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 95, March 1998 (1998-03), pages 2216-2221, XP002112685	1,10
Y	abstract; figures 2-5 page 2217 page 2219, paragraph 4 page 2220, paragraph 3 -page 2221, paragraph 1	2-9

-/--

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14 July 2000	21/07/2000

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	DE KRAKER, JAN-WILLEM ET AL: "(+)-Germacrene A biosynthesis: The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory." PLANT PHYSIOLOGY (ROCKVILLE), (AUG., 1998) VOL. 117, NO. 4, PP. 1381-1392., XP002112686 the whole document	2-9